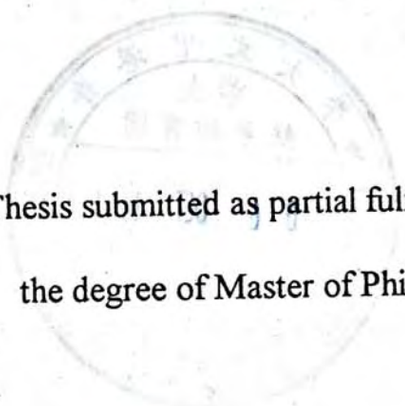
Intergeneric hybridization of *Schizophyllum commune* and *Pleurotus florida* by Protoplast fusion

by

To Siu-Wing



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To my parents

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ABSTRACT

Intergeneric hybridization of the two auxotrophic mutant strains, *Pleurotus florida* and *Schizophyllum commune,* was carried out. Firstly, the specific conditions for protoplast release of the two fusion parents were studied. Protoplast fusion of the two fusion parents using a polyethylene glycol solution was then carried out. So far, three fusion products were obtained from the protoplast fusion experiment.

The optimal conditions for producing mycelial protoplasts from *P. florida* and *S. commune* was developed. For *P. florida,* the maximum yield of protoplasts was obtained with 2.2 % w/w Novozyme234. When 5 days old mycelium was incubated with this enzyme solution in 0.8 M mannitol solution at 28 ^{o}C, 5×10^{7} protoplasts per 300 mg mycelium (wet weight) were induced within 90 minutes. For *S. commune* maximum yield of protoplasts was obtained with enzyme mixture of 0.5 % Novozyme234 and 1.8 % Lywallzyme. When 15 hours old mycelium was incubated with this enzyme solution in 0.8 M mannitol solution at 28 ^{o}C, 8×10^{6} protoplasts per 200 mg mycelium (wet weight) were induced within 150 minutes. However, protoplast regeneration frequencies for *P. florida* and *S. commune* were found to be 0.65 % and 0.47 % respectively.

Cytological, morphological, physiological and genetical characterization were carried out for both fusion parents and fusion products. The fusion products were classified into two types according to their karyotic stage. The first type of fusion product showed to be a dikaryon (PS1) and the second type showed to be monokaryons (PS2 and PS3). The dikaryotic fusion product formed fruit body with pileus, gill and sterigmata morphology similar to that of *S. commune* but have

a long stipe and showed to be a leaky adenine requiring mutant. Progeny analysis showed the reoccurrence of nicotine requiring phenotypes in the first generation progeny of PS1. Mycelial morphologies of the two monokaryotic fusion products were similar to either of the two fusion parents. All the second type fusion products showed to be prototrophs. All three fusion products had their growth rate faster than that of the morphologically similar fusion parent. Results of drug resistance tests showed that all of them became more resistance to acriflavin relative to the two fusion parents. Although PS1 was shown to be adenine requiring, results of progeny analysis showed the presence of nicotine requiring phenotype in 25 to 28 % single spore isolates. AP-PCR fingerprinting of the five strains showed that one of the four DNA profiles of PS1 showed high similarity with the corresponding one of *S. commune*. For PS3, two of the four DNA profiles showed high similarity to that of *P. florida*. The accumulated evidence suggested the possibility that some form of genetical changes may be taken place in the fusion products through intergeneric protoplast fusion of the two fusion parents.

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ABBREVIATIONS

A_{260}	:	Absorbance at wavelength 260 nm
Acr	:	Acriflavin [SIGMA]
BMF	:	Back mutation frequency
CsCl	:	Caesium chloride *[MERCK]
CM	:	Complete medium
CM_{Acr}	:	CM supplemented with Acriflavin
CM_{Gua}	:	CM supplemented with guaiacol
DAPI	:	4',6-diamidino-2-phenylindole [SIGMA]
DC	:	Direct current
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxynucleoside triphosphate [PERKIN ELMER CETUS]
E.C.	:	Enzyme concentration
EDTA	:	Disodium ethylenediaminetetraacetate·$2H_2O$ [MERCK]
Gua	:	Guaiacol [SIGMA]
HCl	:	Hydrochloric acid [MERCK]
MM	:	Minimal medium
MMade	:	MM supplemented with adenine
MMnic	:	MM supplemented with nicotine
MMade,nic	:	MM supplemented with adenine and nicotine
MYG	:	Malt extract - Yeast extract - Glucose medium
NaOAc	:	Sodium acetate [SIGMA]
PCR	:	Polymerase chain reaction
PDA	:	Potato dextrose agar [BIOLIFE]
PDB	:	Potato dextrose broth [DIFCO]
RF	:	Regeneration frequency of protoplasts

RNAase	:	Ribonuclease [SIGMA]
RS	:	Resuspending solution
SDS	:	Sodium dodecyl sulfate [SIGMA]
TBE	:	Tris-Borate-EDTA buffer
TE	:	Tris-EDTA buffer
Tris	:	Hydroxymethyl aminomethane [SIGMA]

*REMARK : [ ] - Brand name of chemical.

Part I

General Aspects

Chapter 1

General Considerations

The increasing world population as well as energy shortage necessitates the production of food in a cheaper and simpler way. Edible mushrooms are a kind of domesticated food which can be cultivated at low cost. At present, the three most popularly cultivated mushrooms are *Agaricus*, *Lentinus* and *Volvariella*. However, the *Pleurotus* mushrooms show an increasing demand in several countries. The *Pleurotus* mushrooms show to be another types of attractive edible mushrooms besides those mentioned above because of their ability to grow on a variety of agricultural and industrial wastes, such as paddy straw, corn cob, cotton waste, cotton seed hull and sawdust. Not to say the recycling of this wastes by the mushroom benefits the whole world on the environmental concerns, the cost of cultivation becomes much more reduced. In addition, the growing conditions of the *Pleurotus* mushrooms do not require to be controlled strictly. Therefore, this variety of mushroom has a prominent prospect to be consumed as the more essential food products. Besides the increasing consumption of *Pleurotus* mushrooms, more new edible mushrooms such as the *Dictyophora duplicata* and *Tremella fuciformis* are now successfully cultivated (Chang, 1982). All these information reflects the need for more varieties of cultivated mushrooms. By the way of founding more potential edible mushroom varieties, strain improvement can also contribute a lot on the aspects concerned above.

Another mushroom named *Schizophyllum commune*, which is a non-edible mushroom, is one of the most intensively investigated basidiomycete with respect to fruit body formation (Wessels, 1985). For example, some studies focused on the genetic and environmental aspects of fruiting in *S. commune* (Raper and Kongelb, 1958) and some focused on mapping of A and B mating-type loci by hybridization (Horton and Raper, 1991). Moreover, there are numerous interesting and detailed information on other aspects of this species. Such as the study of extracellular and intracellular phenol oxidase activity during growth and development (Philips and Leonard, 1976) and some transformation properties of *Schizophyllum commune* (Specht *et al.*, 1991).

Apart from genetic engineering and traditional mutation and selection procedures, manipulation of protoplasts represents the most important approach for strain improvement (Vyskocil, 1987). There are different methods of protoplast manipulation dealing with strain improvement. Protoplast mutagenesis and protoplast fusion are some examples. The former method will not be further discussed here. For the purpose of strain improvement and breeding programmes, protoplast fusion is a recognized method for establishing crosses in fungi (Peberdy, 1987).

On the aspect of protoplast fusion, previous studies involving the two fungi mainly focused on interspecific fusion among different *Pleurotus* species (Toyomasu et al., 1986; Yoo et al., 1984; Yoo et al., 1987a; Yoo et al., 1987b). Heterokaryon formation of *Schizophyllum commune* by electrofusion of protoplasts has also been reported (Sonnenberg and Wessels, 1987). On the other hand, intergeneric protoplast fusion between *Pleurotus ostreatus* and *Ganoderma applanatum* (Yoo et al., 1989) and intergeneric hybridization between *Pleurotus sajor-caju* and

Schizophyllum commune by protoplast fusion (Liang and Chang, 1989) have also been reported.

Successful hybridization of *Pleurotus florida* with *Schizophyllum commune* will made it possible to study the genomic relationships of the parental species and generation of novel hybrids by transfer genetic material between each other. Such advances in both knowledge and technologies may ultimately led to a new way of strain improvement in mushroom. Actually, only a few studies have been reported about intergeneric protoplast fusion. To shed some light on these matters, we have attempted to carry out an intergeneric protoplast fusion experiment of two basidiomycetes, *Pleurotus florida* (Pf67) and *Schizophyllum commune* (Sc17) through PEG-mediated fusion method. Studies on the aspects of the morphologies, nutritional abilities, drug resistance characters and incompatibility reactions of the hybrid strain and its parents, as well as their DNA similarities using genomic fingerprinting technique of arbitrarily-primed polymerase chain reaction were carried out.

CHAPTER 2

Literature Review

2.1. History of fungal protoplast fusion

2.1.1. Fungal protoplast preparation technique

Early in 1892, Klercker, J. von. carried out an experiment on mechanical isolation of plant protoplasts (Gleba and Sytnik, 1984). However, obtaining protoplasts mechanically has the major disadvantage of low efficiency. Enzymatic isolation of protoplast was not developed until early 1950's, . After the elaboration of the technique, improvement of the method to produce large quantity of isolated protoplasts became possible. First enzymatic isolation of protoplast was carried out by Tomcsik, J. and Guex-Holzer, S. in 1952 (Marquis and Coner, 1976). Protoplasts were isolated from bacteria, *Bacillus* cells by lysozyme action. The resulting protoplasts lost their characteristic rod shape and became osmotically sensitive. Through this experiment, the osmotic protection and cell shape maintaining function of thecell wall was clarified. Since then scientists tried to explore the potential of using the enzymatic cell wall degradation technique for other cell wall bounded organisms in order to produce the corresponding protoplasts for different biotechnological applications. Until 1958, first fungal protoplast were successfully produced from *Neurospora crassa* by using crude gut extract of the snail *Helix pomatia* to remove the cell wall (Emerson and Emerson, 1958). From the insight of Emerson and Emerson's report, some scientist might presciently foresee the brilliant prospects of the applications of fungal protoplasts in various areas. Therefore, large amount of studies were carried out on the aspects of enzymatic isolation of protoplasts from various fungal species (Table 2.1.).

Table 2.1. Some examples of fungi from which protoplasts have been isolated.

Fungus	Cell wall degradating enzyme source	Reference
Rhizopus nigricans	*Helix pomatia*	Gabriel, 1968
Mortiella vinacea	*Streptomyces*	Peberdy, 1971
Schizophyllum commune	*Trichoderma viride*	de Vries and Wessels, 1972
Cephalosporium acremonium	Gytophaga prep.	Fawcett *et al.*, 1973
Aspergillus flavus	*Trichoderma viride*	Peberdy *et al.*, 1976
Claviceps purpurea	β-Glucuronidase	Keller *et al.*, 1980
Trichoderma reesei	Driselase	Picataggio *et al.*, 1983
Pleurotus florida	β-D-Glucose and Novozyme234 and snail enzyme	Yoo *et al.*, 1984
Agaricus bisporous, Volvariella volvacea	Novozyme234 and Lywallzyme	Chang *et al.*, 1985
Corprinus macrorhizus	Chitinase and cellulase and glucanase	Yanagi *et al.*, 1985
Lentinus edodes	Cellulase and chintinase	Kawasumi *et al.*, 1987
Flammulina velutipes	Novozym 234 and cellulase CP	Yea *et al.*, 1988

2.1.2. Application of fungal protoplasts

As a matter of fact, the development of protoplast isolation technique enables scientists to step from the organism level down to cellular level and making feasible

experiments on millions of cells in a test tube. Therefore, protoplasts have been used as aids in the study of biochemical as well as physiological processes of fungal metabolism. For example, studies on metabolism of *Cephalosporium acremonium* and *Penicillum chrysogenum* protoplasts has showed that both the respiratory activity and amino acid pools as well as the antibiotic production were similar to those of control mycelia (Fawcett *et al.*,1973). Moreover, it was found that the transport system during protoplast isolation of *C. acremonium* was loss because the uptake of valine was lower in protoplasts than mycelia. The studies of Fawcett *et al.* (1973) also reported the production of secondary metabolites from fungal protoplasts, that is, producing Cephalosporin C and Penicillin N from protoplasts of *C. acremonium* and *Penicillum chrysogenum* respectively. Production of β-galactosidase from *Sclerotium rolfsii* protoplasts was reported by Deshpande *et al.* in 1987. Besides using fungal protoplasts for the production of compounds such as antibiotics or enzymes as mention above, protoplasts have also had a major impact on fungal genetics (Peberdy, 1987). With the development of fusogen for fusion of plasma membrane, cell fusion became possible (Lucy, 1970). Since the production of protoplasts from fungi removed the physical barrier of incompatibility, protoplasts isolation technique able to remove such barrier and enable protoplasts from different strains to be fused by fusogen treatment. The first induced protoplast fusion was carried out with plant protoplasts by using sodium nitrate as fusogen (Power et al., 1970). No report has been described on the application of induced fusion techniques to fungal protoplasts until 1974 (Ferenczy *et al.*, 1974). Afterwards, the conditions necessary to achieve high fusion frequencies using polyethylene glycol have been extensively investigated by Anné and Peberdy (1975, 1976) using *Penicillium chrysogenum*. Electrofusion of protoplasts has also been reported by Zimmerman in 1982. However, electrofusion techniques suffered the major limitation of scale (Peberdy, 1987). With the advances in polyethylene glycol fusion techniques, intraspecific, interspecific and intergeneric crosses have been reported for a number

of basidiomycetes strains (Table 2.2). According to Peberdy (1987), protoplast fusion techniques mainly found to be applied for the researches with the objective to generate novel hybrids or genetics studies such as heterokaryon incompatibility. Moreover, protoplast fusion techniques have already become an essential step to breeding based on its potential in establishing parasexual crosses.

As shown in Table 2.2., generation of novel hybrids by protoplast fusion of *Pleurotus cornucopiae* and *Lentinus edodes* have been reported recently (Ogawa, 1993). The hybrids showed to have accelerated growth rate and was able to produce *Lentinus*-like fruit body in half cultivation time relative to that of the *Lentinus* parent. On the other hand, experiments on intraspecific protoplast fusion of mutant strains of *Aspergillus nidulans*, incompatible *Verticillium* strains, and *Cochliobolus heterostrophus* strains revealed different levels of incompatibility, i.e., hyphal wall, cytoplasmic or nuclear level (Peberdy, 1987). On the aspect of the parasexual process in fungi, intraspecific fusion products of *Trichoderma reesei* have been proved to be useful in studying the process. Through assessment on the DNA content of conidia and spores of the fusion products, the results showed to be able to provide good evidence for the occurrence of diploid phase and subsequent haploidization in parasexual cycle of fungi (Manczinger and Ferenczy, 1985).

Table 2.2. Some examples of intraspecific, interspecific and intergeneric protoplast fusion applied to basidiomycetes.

Fungus	Reference
Intraspecific fusion	
Coprinus macrorhizus	Kiguchi and Yanagi, 1985
Pleurotus ostreatus	Ohmasa, 1986
**Schizophyllum commune*	Sonnenberg and Wessels, 1987
Coprinus cinereus	Yanangi *et al.*, 1988
Oudemansiella mucida	Homolka, 1988
Pleurotus salmoneostramineus	Toyomasu and Mori, 1989; Iijima *et al.*, 1991
Interspecific fusion	
Pleurotus ostreatus × *P. florida*	Yoo *et al.*, 1984; Yoo *et al.*, 1986
Pleurotus ostreatus × *P. salmoneostramineus*	Toyamasu *et al.*, 1986
Pleurotus sajor-caju × *P. columbinus*	Toymasu and Mori, 1987
Ganoderma applanatum × *Ganoderma lucidum*	Park *et al.*, 1988; Um *et al.*,1988
Pleurotus ostreatus × *Pleurotus columbinus*	Toyomasu and Mori, 1989
Intergeneric fusion	
Pleurotus sajor-caju × *Schizophyllum commune*	Liang and Chang, 1989
Pleurotus cornucopiae × *Lentinus edodes,*	Ogawa, 1993
Pleurotus cornucopiae × *Lyophyllum decastes*	

* - Protoplast fusion was carried out by electrofusion techniques.

2.2. Protoplast fusion by polyethylene glycol (PEG)

In the last decade, cell fusion induction is an area of research that has been developed extensively. A number of approaches, such as chemical, electric field and virus, are now known to have the ability to induce cell fusion. Considering the application of protoplast fusion techniques in production of somatic hybrids, polyethylene glycol (PEG) is the widely used chemical fusogen for inducing cell fusion in fungi (Peberdy, 1987). Through microscopic observations, protoplasts were found to be shrink and aggregate during PEG treatment. Unlike electrofusion, which allow controlled cell fusion by micromanipulation of two point-adherence protoplasts (Senda *et al.*, 1979), PEG-induced protoplast fusion is a random event (Peberdy and Fox, 1993). So far, the mechanism of PEG-induced protoplast fusion is still not fully understood.

Microscopic observations showed that in the present of PEG, protoplasts showed to be dehydrated and aggregated (Kiguchi and Yanagi, 1985). These effects of PEG were important for maintaining protoplasts in close proximity and hence fusion of plasma membrane of two or more protoplasts was allowed. In order to resolve the principle behind the aggregation effect, dehydration and fusion induction of PEG on protoplasts, extensive studies on membrane lipid stability in PEG were carried out. On the aspect of the changes on the arrangement of membrane lipid, the stability of lipid bilayers was found to be dependent on the attractive and repulsive forces in the adjacent membrane bilayer system. (LeNeuve *et al.*, 1977). These forces involved the cohesive force between phospholipids and water, the electrostatic repulsive forces between adjacent bilayers, and the van der Waals attractions between the hydrocarbon chains. Maggio *et al.* (1976) reported that surface potential of phospholipid bilayers was decreased by increasing the PEG 6000 concentration from 0.5 to 5 %. Hence, the electrostatic repulsive force between

bilayers was being reduced. McIver (1979) stated that the steric hydration forces were thought to be the primary barrier to membrane fusion. The result of his study showed that the vesicle aggregations were consistent with the ability of PEG to bind water and removed the hydration shell surrounding lipid molecules. The direct interaction of PEG with membrane phospholipids to change surface potential and compete with the available water weakened the association between the phospholipids in the bilayer. For triggering the fusion of protoplasts, the binding of divalent cations (mainly Ca^{2+}) onto the negatively charged lipid head groups led to the local destabilization of the bilayer was resulted (Raudino and Bianciardi, 1990). The destabilized bilayer then rearranged itself passing through some ill-defined intermediates, eventually led to the merging of the two adjacent bilayers.

2.3. Incompatibility system in fungi

As discussed in the above section, the major breakthrough of the protoplast fusion technique over the conventional breeding programmes, which depends on hyphal fusion of the two parent strains, was the ability of the former techniques to overcome the cell wall level incompatibility between the parental strains. However, cytoplasmic and nuclear level incompatibility between the two fusion parents were expected to be exit in the resulting somatic hybrids after protoplast fusion process. Nowadays, we have some knowledge of the events, such as nuclear migration and conjugate nuclear division, involved in the formation of the dikaryon between compatible homokaryons of the same species as well as the exiting of mating-type loci(us) which govern the compatibility interaction development in higher fungi (Novotny *et al.*, 1991). The most elaborate mating - type genes are found in the Basidiomycotina, with most containing two different A and B loci (Ashby and Dyer, 1992). These were though to involve a total of four genes. However, Casselton and

Kües (1992) reported that the regions were likely to be far more complex, with at least seven genes identified in the ink cap *Coprinus cinereus*.

Actually, we still have a lot of questions on the aspect of compatibility interactions, such as the molecular basis of the self and non-self recognition, to address (Casselton and Kües, 1992). Since this is intracellular, it is likely to involve an interaction between the products of mating-type loci. However, it still remains a mystery on the aspect of the protein - DNA interactions involved in the gene regulation of the compatibility interaction (Novotny *et al.*, 1991). Although there is still a long way to go for resolving those questions in the compatibility process, somatic hybrids between incompatible strains were successfully generated by protoplast fusion techniques. For example, the interspecific hybridization between species of *Aspergillus* which would not hybridize by any natural means was able to generated hybrid with novel phenotype (Kevei and Peberdy, 1984). For basidiomycetes, interspecific protoplast fusion was carried out extensively with *Pleurotus* (Yoo *et al.*, 1984; Yoo *et al.*, 1986; Toyamasu *et al.*, 1986; Toyomasu and Mori, 1987) and Ganoderma (Park *et al.*, 1988; Um *et al.*,1988). In addition to the potential of interspecific protoplast fusion techniques for generating novel hybrids, it also provided an approach for understanding the underlying interaction of mating type genes in these hybrids and may eventually lead to a new interpretation on the taxonomic relationships of some species. Toyomasu and Mori (1987) attempted to carry pairwised protoplast fusion between five *Pleurotus* species, *P. columbinus*, *P. pulmonarius*, *P. ostreatus*, *P. sajor-caju*. They reported that only the interspecific crosses between *P. ostreatus* and *P. columbinus*, and between *P. pulmonarius* and *P. sajor-caju* led to clamp connection formation. Therefore, the designation of several supposed species is rather doubtful. On the aspect of incompatibility interaction, Peberdy and Fox (1993) suggested that the potential for interaction of the two genomes might be possible if the mating type gene and the corresponding products in

these hybrids were highly conserved. Therefore, the lack of recognition of cell wall may be the major barrier for initiation of hyphal fusion in the corresponding mating crosses.

Potentials on the application of protoplast fusion techniques in somatic hybridization are not limited to the extent between different species, somatic hybridizations between fungal strains in different genera have also been reported (Table 2.2). So far, only a few reports on intergeneric protoplast fusion of basidiomycetes were published. However, generation of novel hybrids between basidiomycetes further elaborate the possibility of conservative nature of mating type genes suggested by Peberdy and Fox (1993) and it may even open up a new area for interpretation of the taxonomic relationships of some genus.

2.4. Characterization of fusion products by genetic markers

In crosses of protoplast fusion, both genetical and biochemical analysis of the fusion products are essential (Peberdy and Fox, 1993). Therefore genetic markers become important in characterization of fusion product. A genetic marker is defined as an allelic difference occurring at a certain position (locus) on a chromosome. Such difference makes it possible to follow the transmission of that locus through successive generations (Chang, S. T., personal communication). There are many different types of genetic marker have been used for characterization of fusion products in previous protoplast fusion systems. Some examples are the morphological characters (Ogawa, 1993; Stasz *et al.*, 1988; Toyomasu and Mori, 1987; Kirimura *et al.*, 1989), isoenzyme profile (Toyomasu, *et al.*, 1986; Toyomasu and Mori, 1987), DNA content (Ogawa *et al.*, 1988; Kirimura *et al.*, 1989), specific enzyme activity (Pina *et al.*, 1986). However, the coexisting of the parental genetic materials could only be proved indirectly by these markers. Moreover, some of these

markers, such as the isoenzyme profile or morphological characters, may vary with changes in experimental conditions.

As characterization of fusion can be difficult when only relied on only one of the markers based on morphological characters or DNA content or even some change in metabolic changes as mentioned above, the development of molecular markers based on qualitative changes of DNA for identification of fusion products could greatly increase the usefulness and attractiveness of protoplast fusion as a technique for fungal breeding programme. The use of pulse field gel electrophoresis could provide two kinds of molecular markers, electrophoretic karyotype and restriction fragment length polymorphisms (RFLP) (Jong and Birmingham, 1991). Actually, RFLP has already applied to discriminate different strains of *Coprinus cinereus* from different origins early in 1983 (Wu *et al.*, 1983). On the aspect of electrophoretic karyotyping, the number of bands and genomic sizes of four different strains of *Pleurotus florida* were showed to be different. The number of bands for the four strains varied from five to eight and the total chromosomal DNA size ranged from 18.2 to 34.99 Megabase pairs (Peberdy and Fox, 1993). Therefore, changes in the genome of fusion products relative to the fusion parents may be assessed by using these molecular markers. In recent years, rapid development of polymerase chain reaction (PCR) technique also showed to be a potential approach for characterization of fusion product. Both arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh and McClelland, 1990) and the random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990) can generate DNA product for genomic fingerprinting. The potential of AP-PCR fingerprinting in characterization of a putative protoplast fusion product has been reported by Chiu *et al.*(1993). More information on the application of AP-PCR will be given on chapter 7.

Part II

Optimization of Protoplast Release and Protoplast Fusion Studies

CHAPTER 3

Protoplast Isolation of *Pleurotus florida* (Pf67) and *Schizophyllum commune* (Sc17)

3.1. Introduction

In order to carry out a successful protoplast fusion experiment, optimization of protoplast isolation seems to be a prerequisite. Although the methods for preparing protoplasts from filamentous fungi are well established (Peberdy, 1979a and Davis, 1985), the protoplast isolation condition is idiosyncratic for each fungus (Deed and Seviour, 1990). Therefore, parameters affecting the protoplast isolation were studied in this chapter.

Parameters involved in optimization include: choice of lytic enzyme, duration of enzyme digestion , effect of culture age, as well as the type and concentration of osmotic stabilizer. The following series of experiments were mainly focused on all the above parameters with an aim to optimize the conditions for maximum protoplasts yield of the two strains : *Pleurotus florida* and *Schizophyllum commune*, and, although determined separately, most interact to affect the overall yield.

3.2. Materials and methods

3.2.1. Strains and medium

Schizophyllum commune (Sc17) and *Pleurotus florida* (Pf67) were used as the auxotrophic mutant strains. Both strains have their corresponding genetic markers as described in table 3.1..

Table 3.1. Available markers of *Schizophyllum commune* (Sc17) and *Pleurotus florida* (Pf67).

Available markers	*Schizophyllum commune* (Sc17)	*Pleurotus florida* (Pf67)
Auxotrophic marker	Adenine requiring (ade^-) Nicotinic acid requiring (nic^-)	Adenine requiring (ade^-)
Drug resistance marker	Acriflavin (100 μg/ml) resistance Guaiacol (1 μg/ml) resistance	Acriflavin (100 μg/ml) sensitive Guaiacol (1 μg/ml) sensitive and stains guaiacol agar brown

Potato dextrose agar medium (BIOLIFE) was used as the complete medium for maintaining cultures. MYG liquid medium composed of 10 g/L malt extract (DIFCO), 4 g/L Bacto-yeast extract (DIFCO) and 4 g/L glucose (BIOLIFE) was used to culture mycelia for protoplast isolation (Toyomasu and Mori, 1987). Method for determining the type and concentration of lytic enzyme and osmotic stabilizer as well as culture age for protoplast isolation will be determined separately in section 3.2.2., 3.2.4., 3.2.5. and 3.2.3. respectively.

3.2.2. Protoplast isolation in different types and concentrations of cell wall lytic enzymes

Mycelia growing on PDA agar was placed into 20 ml sterilized distilled water inside a waring blender cup and blended at high speed for 25 seconds twice. For 20

ml MYG liquid medium in a 250 ml conical flask, 5 ml homogenate was inoculated. The culture was then incubated at 28 °C without shaking until a thin mycelial mat was formed. Mycelia were then collected by filtration using a stainless steel wire filter with 0.25 mm^2 grid size. The collected mycelia were blot dried by sterilized filter paper. Three hundred milligrams wet weight of mycelia were placed into 2 ml cell wall digesting enzyme solution, which contained cell wall lytic enzyme in 0.05 M Sorensen's phosphate buffer at pH 5.8 with 0.8 M mannitol as osmotic stabilizer. The mycelia-enzyme solution mixture was incubated in 28 °C and the amount of protoplasts released at each time interval was assessed by counting under microscope using haematocytometer. All statistical data analysis was carried out by student t-test (independent) of Sigma Plot (version 5.0) at P value = 0.05 level. The time of incubation for mycelial mat formation and the concentration of enzyme(s) will be stated clearly in each of the following experiment. For each recorded data point of the following experiments, three protoplasting solutions were produced for each condition as triplicates.

For Pf67, preparation of mycelium and protoplast release was carried out by the method described above. The culture was incubated in MYG for 72 hours. The protoplast yield at six different concentrations of Novozyme234 (14, 16, 18, 20, 22 and 24 mg/ml) was determined. Amount of protoplast released at each 30 minutes' interval through the 210 minutes time course were counted.

For Sc17, the culture was incubated in MYG for 20 hours. The study was divided into three parts. The first part was focused on studying the protoplast yield at four different concentrations of Novozyme234 (4, 5, 6 and 7 mg/ml). The second part compared the combining effect of 5 mg/ml Novozyme234 and 9 mg/ml Lywallzyme with the effect of using either enzyme alone. The third part

investigated the optimum amount of Lywallzyme needed for mixing with 5 mg/ml Novozyme234 to obtain the maximum protoplast yield. The Lywallzyme concentrations series studied was 0, 9, 12, 15, 18, and 21 mg/ml. All other protoplast release conditions were the same as that of Pf67.

3.2.3. Protoplast isolation using mycelia of different culture ages

The preparation of mycelium and procedures for protoplast release were similar to the method described in section 3.2.2.. However, from the results of section 3.2.2., 5 mg/ml Novozyme234 with 9 mg/ml Lywallzyme was used for protoplast release of Sc17 and 22 mg/ml Novozyme234 was used for that of Pf67. This section of experiment was divided into two parts. Part 1 studied the efficiency of protoplast release of both Pf67 and Sc17 through the incubation time course of 1 to 8 days. Amount of protoplast released by the mycelia sample from each 24 hours' interval was counted. The specific type and combination of cell wall lytic enzyme for each of the two strains were corresponded to the conditions established in section 3.2.2.. Part 2 was focused on determining the optimum culture age of Sc17 for protoplast release. For this part of study, the whole time course was 18 hours and mycelia sampling was carried out at each 3 hours' interval.

3.2.4. Protoplast isolation in different types and concentrations of osmotic stabilizers

The protoplast release procedures of Pf67 and Sc17 was similar to that of section 3.2.3. but carried out with two different osmotic stabilizers separately. The culture ages for the two strains used were the same as the optimized results of section 3.2.3. experiment, which were 5 days and 15 hours respectively. They were

mannitol and magnesium sulphate. The concentration ranges of these two osmotic stabilizers studied were 0.4, 0.6, 0.8, 1.0, 1.2 M. Other conditions, such as the specific type and combination of enzyme, the time for enzyme digestion as well as the culture age was the same as those described in the results of section 3.2.2. and 3.2.3..

3.2.5. Collection of protoplasts by centrifugation

After knowing the optimal concentrations of the two osmotic stabilizers for the two strains form section 3.2.4., the efficiency of collecting protoplast of the two strains in the two osmotic stabilizers were studied. Protoplasts obtained from 3.2.4. in 2 ml of osmotic stabilizer were spun down at 1,400 g for 10 minutes by Hettich EBH 3S table top centrifuge. The supernatant was discarded and the pellet was resuspended into 2 ml of resuspending solution. The resuspending solution (RS) consisted of the same type and concentrations of the original osmotic stabilizer in sterilized distilled water. The amount of protoplasts in the 2 ml osmotic stabilizer solution before and after centrifugation was counted. The percentage of collectable protoplasts by centrifugation (Collectable %) was defined by the following equation :

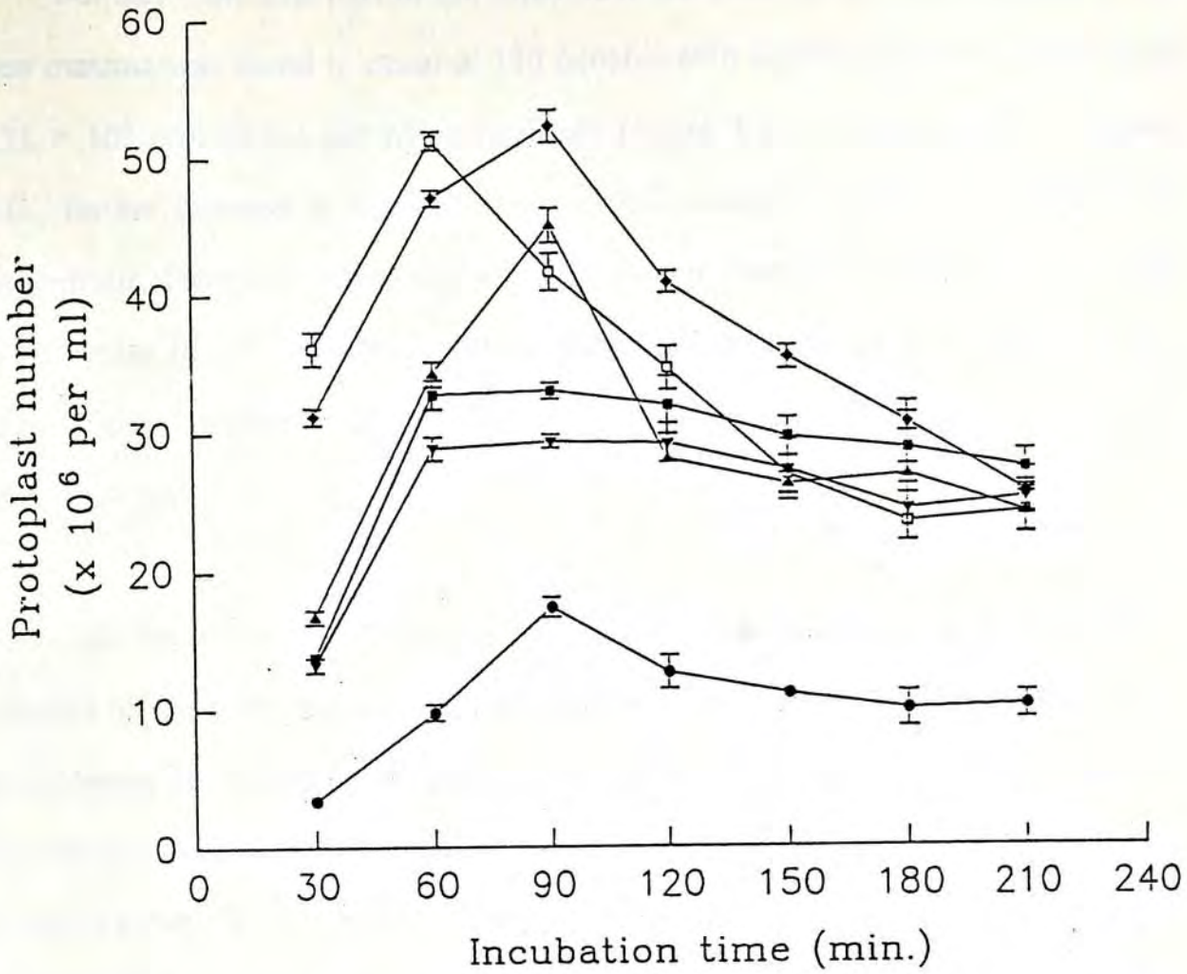
$$\text{Collectable \%} = \frac{\text{Amount of protoplast in resuspending solution}}{\text{Amount of protoplast in osmotic stabilizer solution before centrifugation}} \times 100\ \%$$

The collectable % of protoplasts obtained from the following four conditions described in section 3.2.4. were studied. They are 0.8 M magnesium sulphate and 0.8 M mannitol solution for Pf67 as well as Sc17.

3.3. Results

3.3.1. Effect of types and concentrations of lytic enzyme

The variation of Pf67 protoplast amount with time under different concentrations of Novozyme234 was illustrated in figure 3.1.. For all different concentrations of enzymes, the variation generally showed to have an initial increase with time and then attained a maximum followed by a decrease. Enzyme concentration (E.C.) at 22 and 24 mg/ml showed to have the highest initial protoplast releasing rate for the first 60 minutes. Maximum protoplasts yields (approximately 50×10^6 protoplasts per ml) were obtained at 90 minutes for 22 mg/ml E.C. and 60 minutes at 24 mg/ml E.C.. The two maxima showed to be statistically not different (figure 3.1.). The maxima for other E.C. showed to be significantly lower than that of the former two but also occurred at about 60 to 90 minutes incubation time.



• 14mg/ml, ▾ 16mg/ml, ▪ 18mg/ml, ▲ 20mg/ml
◆ 22mg/ml, ▫ 24mg/ml

Figure 3.1. Effect of Novozyme234 concentration on the amount of protoplast release of Pleurotus florida. I–I is the standard error bar.

t-test analysis

Data points comparison (enzyme concentration mg/ml / time point)	t-values, p-values	Conclusions from comparing the means at the 0.05 level.
22/90 vs 24/60	$7.3995e^{-1}$, $5.0042e^{-1}$	Not different.
22/60 vs 22/90	$-3.9134e^{0}$, $1.7343e^{-2}$	Different.
22/60 vs 24/60	$-4.6423e^{0}$, $9.7167e^{-3}$	Different.
22/60 vs 24/90	$5.8456e^{0}$, $4.2708e^{-3}$	Different.

P = 0.05 level.

For Sc17, the first part of the experiment showed that at 5 and 6 mg/ml E.C., their maxima was found to occur at 120 minutes with a protoplast yield of 0.25 and 0.21×10^6 protoplasts per ml respectively (figure 3.2.). However, for 5 mg/ml E.C., further increase in digestion time to 210 minutes resulted in a statistically insignificant decrease. For 6 mg/ml E.C. , further increase in digestion time from 120 minutes to 150 minutes caused a significant decrease in protoplast amount. Both 4 and 7 mg/ml E.C. showed to have a relatively much lower maximum protoplast yield.

For the second part, figure 3.3. showed that the amount of protoplasts released by the three enzyme systems were significantly different from each other. Considering the maximum protoplast yield by the three enzyme solutions, they can be arranged in a decreasing order: 5 mg/ml Novozyme234 + 9 mg/ml Lywallzyme > 9 mg/ml Lywallzyme > 5 mg/ml Novozyme234. The maximum protoplast yield of the combined enzyme system was found to be five folds greater than the summation of the results of the two single enzyme system.

For the third part, figure 3.4. showed that the increase in Lywallzyme concentration from 9 to 18 mg/ml resulted in a general increase in protoplast yield but the change was statistically insignificant.

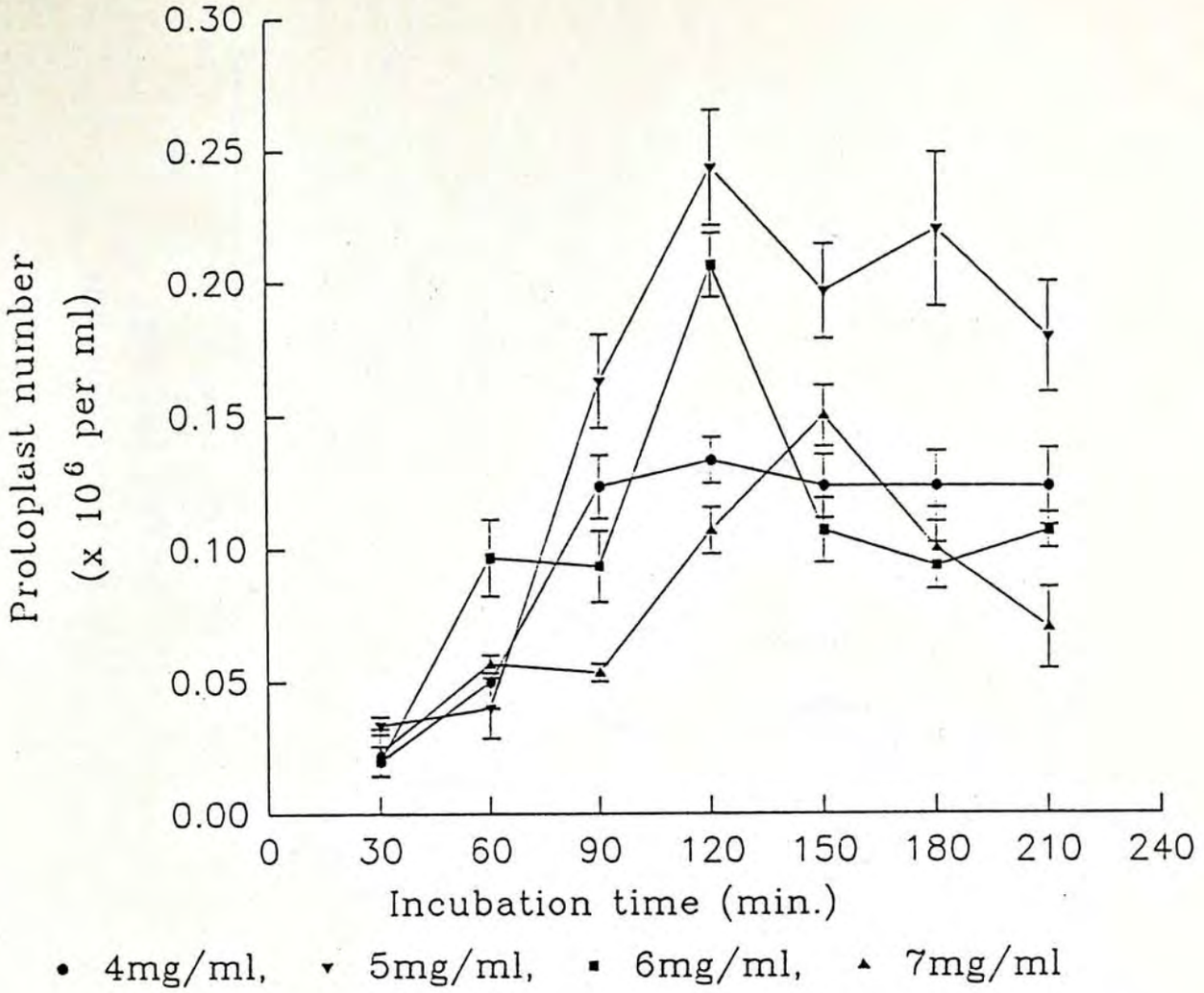
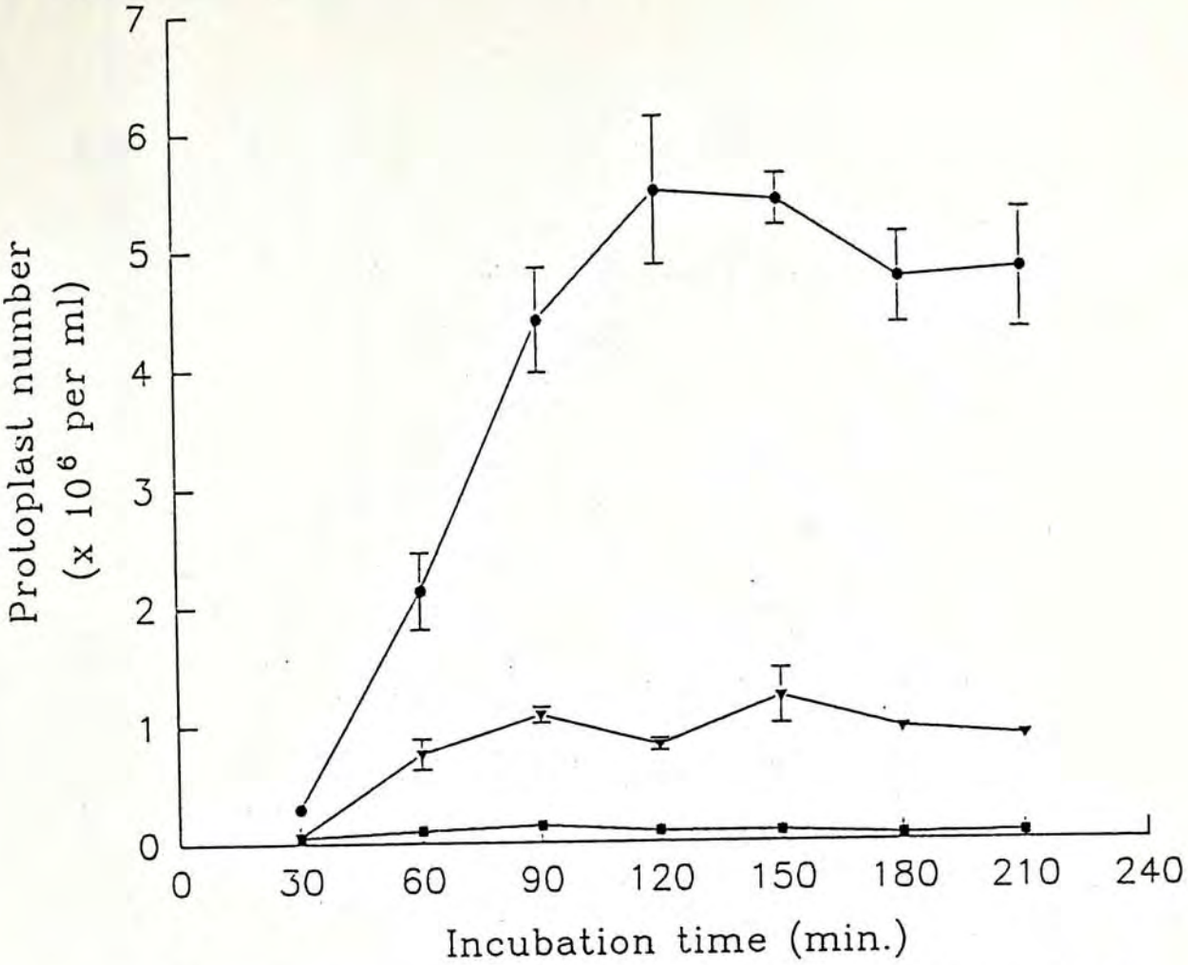


Figure 3.2. Effect of Novozyme234 concentration on the amount of protoplast release of Schizophyllum commune. I–I is the standard error bar.

t-test analysis

Data points comparison (enzyme concentration mg/ml / time point)	t-values, p-values	Conclusions from comparing the means at the 0.05 level.
5/120 vs 6/120	$1.4699e^{0}$, $2.1552e^{-1}$	Not different.
5/120 vs 5/210	$2.0981e^{0}$, $1.6386e^{-1}$	Not different.
6/120 vs 6/150	$5.8834e^{0}$, $4.1714e^{-3}$	Different.

P = 0.05 level.



▪ 5mg/ml Novozyme234, ▾ 9mg/ml Lywallzyme,ml
• 5mg/ml Novozyme234 + 9mg/ml Lywallzyme

Figure 3.3. The independent and combine effect of 5mg/ml Novozyme234 and 9mg/ml Lywallzyme on the amount of protoplast release of Schizophyllum commune.
I–I is the standard error bar.

t-test analysis

Data points comparison (enzyme concentration mg/ml / time point)	t-values, p-values	Conclusions from comparing the means at the 0.05 level.
5N*+9L*/120 vs 9L/150	$6.3994e^{0}$, $3.0621e^{-3}$	Different.
5N+9L/120 vs 5N/90	$9.6791e^{0}$, $6.3755e^{-4}$	Different.
9L/150 vs 5/90	$4.6395e^{0}$, $9.7373e^{-3}$	Different.

P = 0.05 level.
* : N-Novozyme234, L-Lywallzyme.

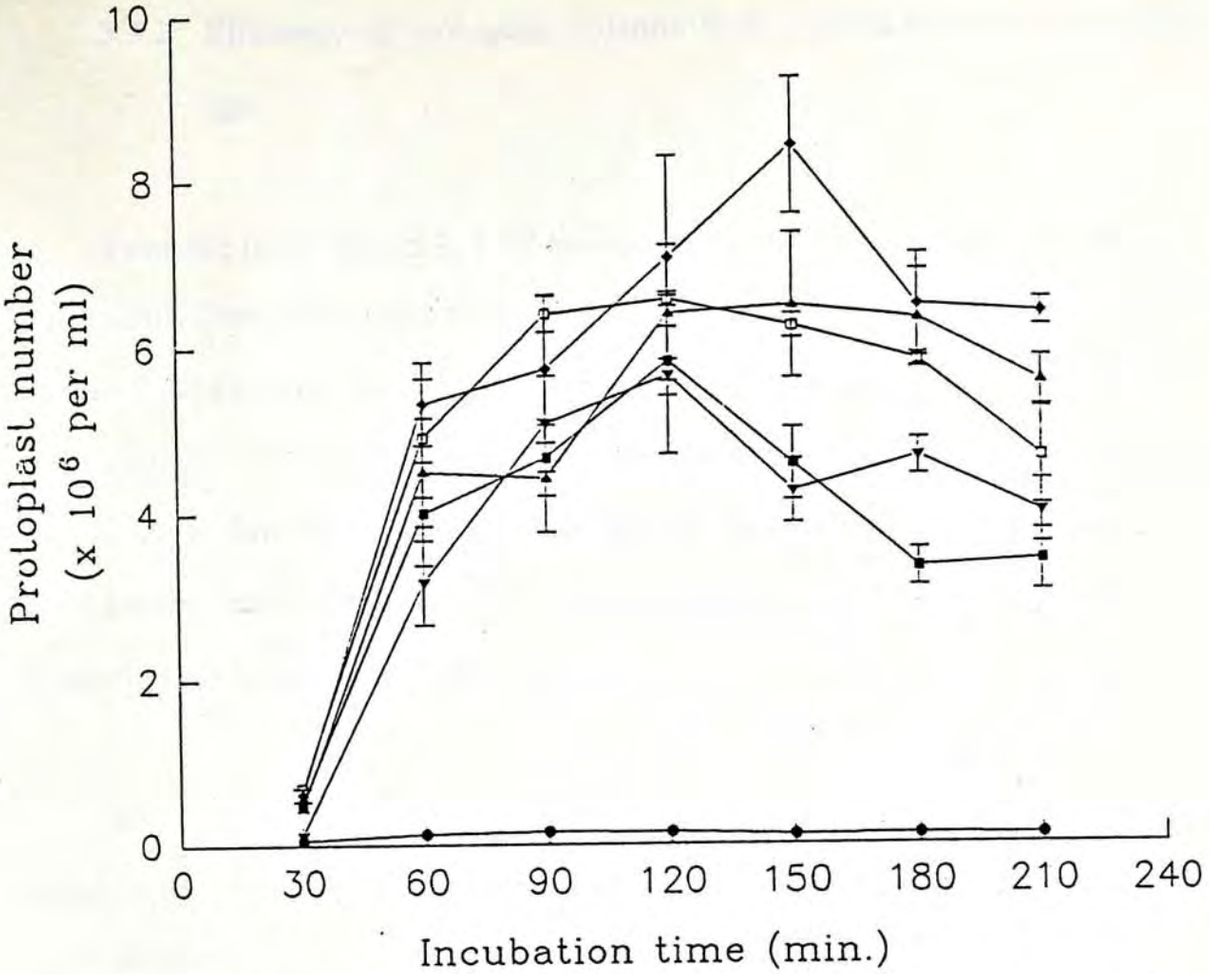


Figure 3.4. Effect of Lywallzyme concentration with 5mg /ml Novozyme234 on the amount of protoplast release of Schizophyllum commune.
I–I is the standard error bar.

t-test analysis

Data points comparison (enzyme* concentration mg/ml / time point)	t-values, p-values	Conclusions from comparing the means at the 0.05 level.
9/120 vs 12/120	$-1.5911e^{-1}$, $8.8123e^{-1}$	Not different.
9/120 vs 15/150	$-6.4018e^{-1}$, $5.5688e^{-1}$	Not different.
9/120 vs 18/150	$-2.1759e^{0}$, $9.5174e^{-2}$	Not different.
9/120 vs 21/90	$-7.6064e^{-1}$, $4.8924e^{-1}$	Not different.

P = 0.05 level.
* : L-Lywallzyme.

3.3.2. Efficiency of protoplast isolation form mycelia with different culture age

From the result of part 1, Pf67 has the protoplast yield showed to be increased significantly from the 3 days to 4 days cultures and decreased significantly from 6 days to 7 days cultures (figure 3.5.). However, the protoplast yield by the 4 days to 6 days cultures showed to be not significantly different form each other which was the highest around 40×10^6 protoplasts per ml. However, for Sc17, the protoplast yield was decreased from the 1 day cultures, which was 7×10^6 protoplasts per ml, to nearly zero for the 2 to 8 days cultures.

For part 2, the protoplast yield of Sc17 showed to be increased from 3 hours cultures to 15 hours cultures (figure 3.6.) then tended to decrease at greater concentrations.

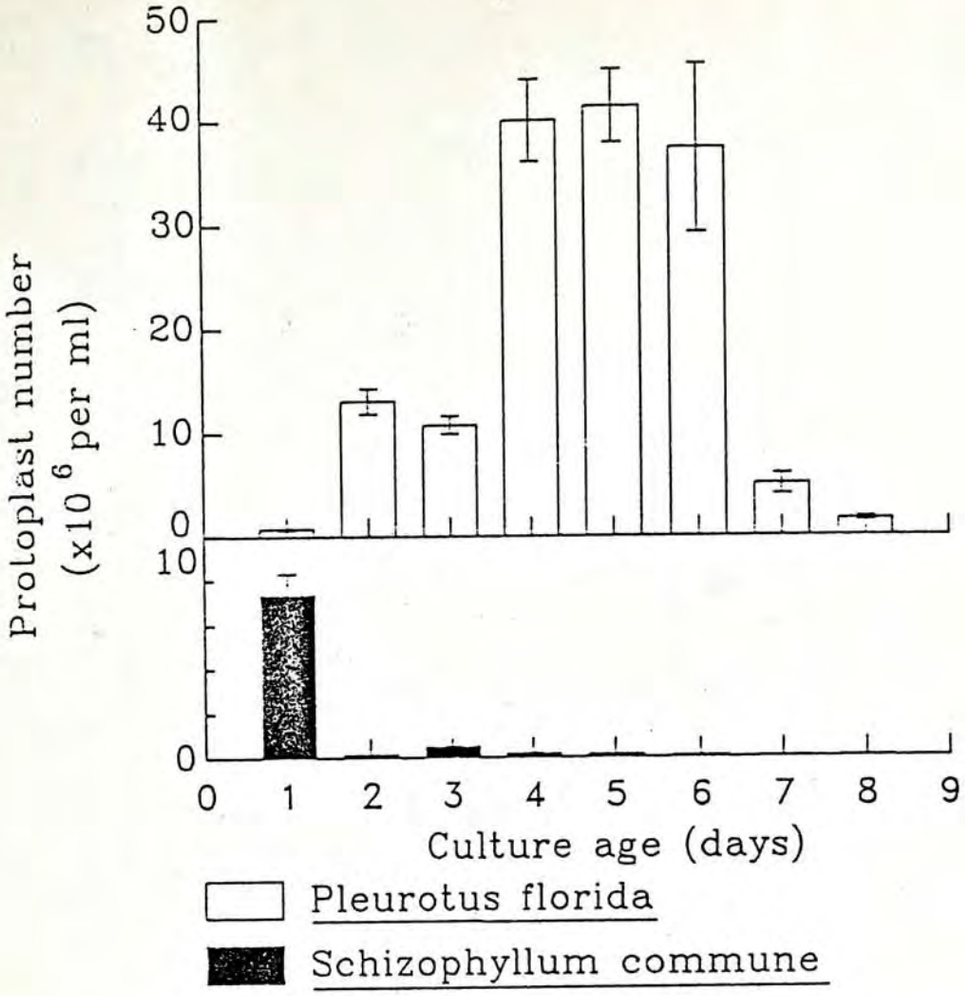


Figure 3.5. Effect of culture age on the amount of protoplast release of Pleurotus florida and Schizophyllum commune. 22mg/ml Novozyme 234 and 90 minutes incubation at 28°C was used for Pleurotus florida. 5 mg/ml Novozyme234 with 18mg/ml Lywallzyme and 150 minutes incubation at 28°C was used for protoplast release of Schizophyllum commune.
I–I is the standard error bar.

t-test analysis

Data points comparison of Pf67 (time point [day])	t-values, p-values	Conclusions from comparing the means at the 0.05 level.
5 vs 4	$2.5000e^{-1}$, $8.2688e^{-1}$	Not different.
5 vs 6	$4.5227e^{-1}$, $6.5127e^{-1}$	Not different
4 vs 6	$2.9488e^{-1}$, $7.6520e^{-1}$	Not different.
4 vs 3	$-7.1420e^{0}$, $3.1797e^{-12}$	Different.
6 vs 7	$-3.9561e^{0}$, $8.9675e^{-5}$	Different.

P = 0.05 level.

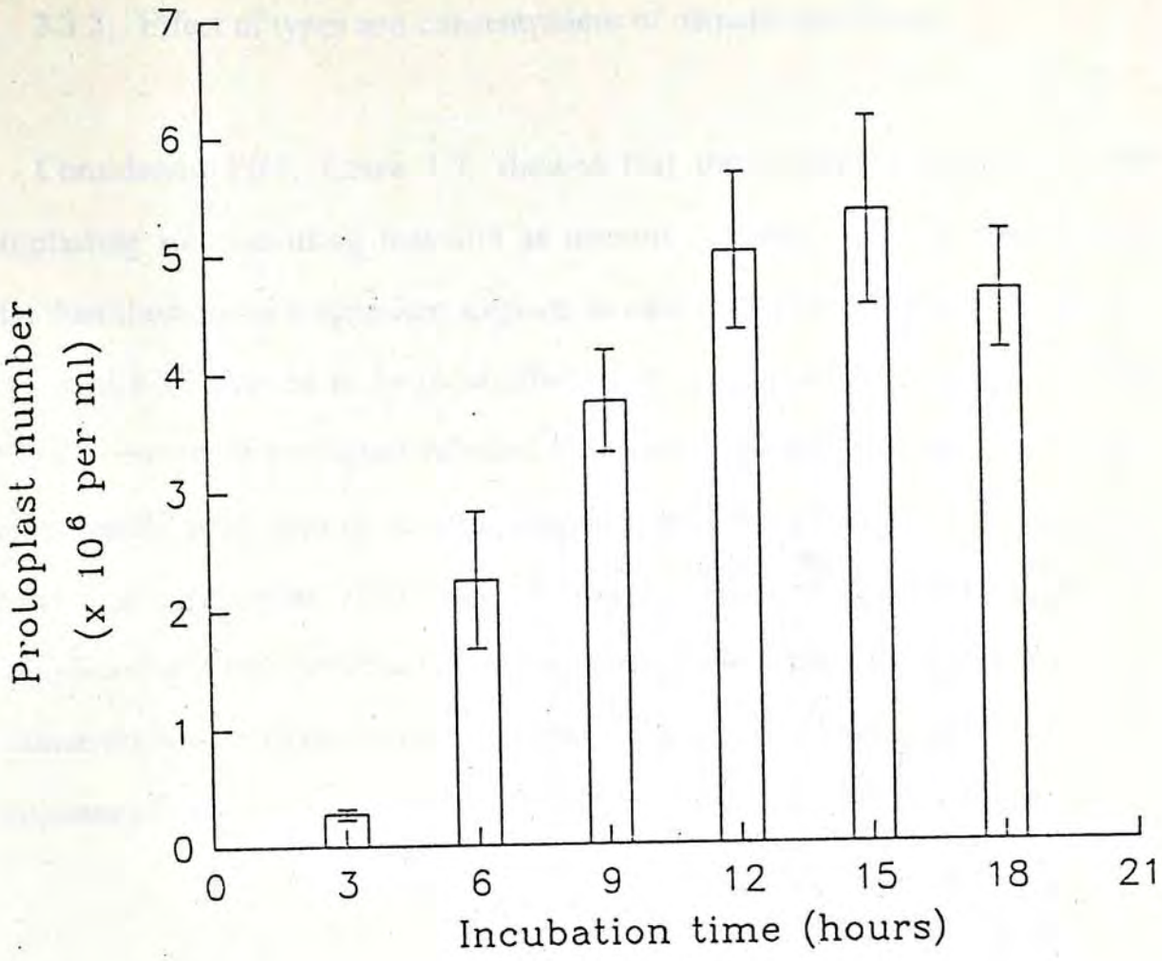


Figure 3.6. The number of protoplast released by the cultures of Schizophyllum commune with different culture ages. 5mg/ml Novozyme234 and 18mg/ml Lywallzyme in 0.8M mannitol and incubation at 28°C for 150 minutes was used for protoplast release. I–I is the standard error bar.

t-test analysis

Data points comparison of Sc17 (time point [hours])	t-values, p-values	Conclusions from comparing the means at the 0.05 level.
15 vs 12	$-3.2233e^{-1}$, $7.6335e^{-1}$	Not different.
15 vs 9	$1.7491e^{0}$, $1.5518e^{-1}$	Not different.
15 vs 6	$3.1383e^{0}$, $3.4905e^{-2}$	Different.
9 vs 12	$-1.5811e^{0}$, $1.8900e^{-1}$	Not different.

P = 0.05 level.

3.3.3. Effect of types and concentrations of osmotic stabilizers

Considering Pf67, figure 3.7. showed that the protoplast yields from the protoplasting solution using mannitol as osmotic stabilizer were greater (2 to 5 folds) than those using magnesium sulphate as osmotic stabilizer. Mannitol solution with 0.8 M E.C. showed to be most effective for protoplast release of Pf67. For Sc17, the amount of protoplast released in mannitol solution were generally lower (approximately 30%) than those using magnesium sulphate (figure 3.8.). The latter had the highest protoplast yield at 1.0 M. For the results using mannitol, there was no significant different between the amount of protoplast released by the 0.8 to 1.2 M concentration and the protoplast yield remained at a high level of 9×10^6 protoplasts per ml.

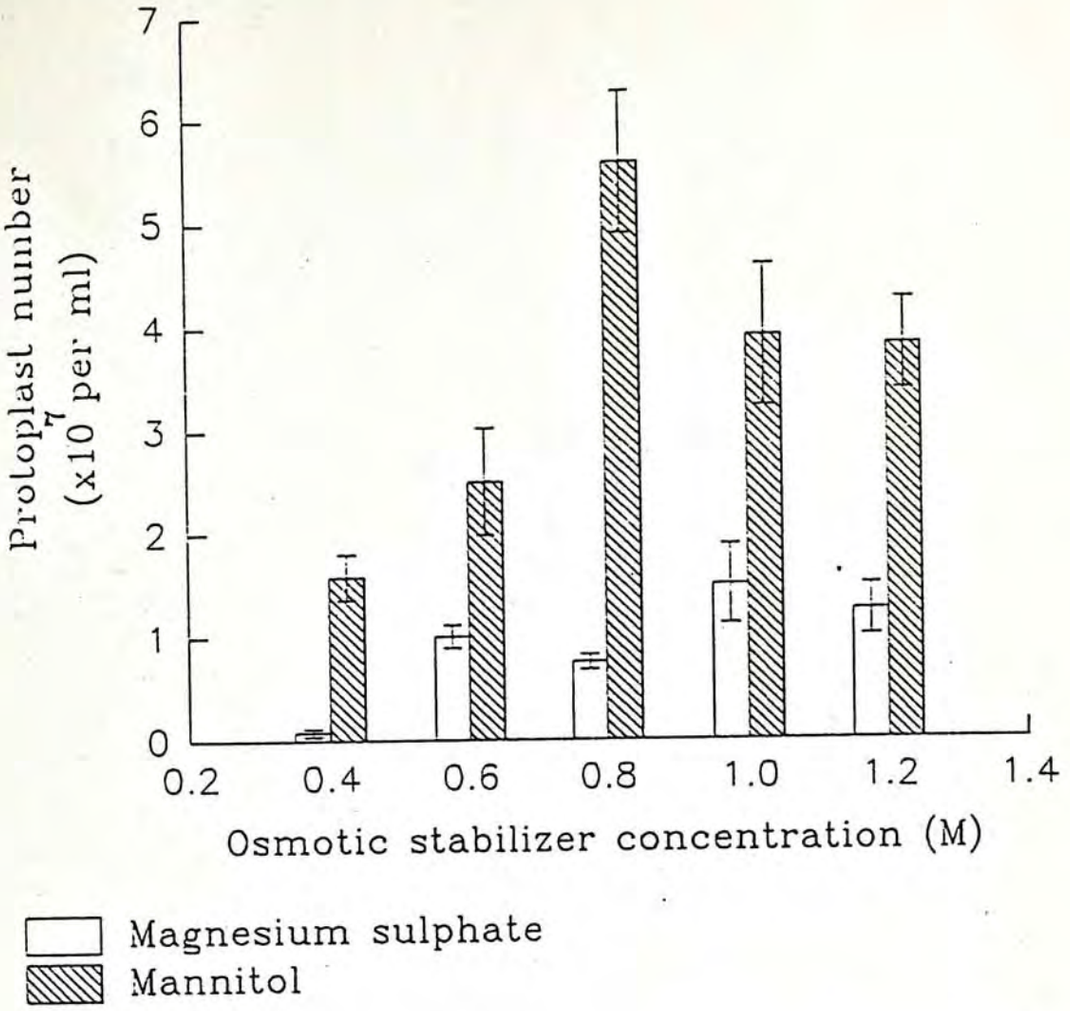


Figure 3.7. Amount of Pleurotus florida protoplast released by two different osmotic stabilizers in different concentrations. 22mg/ml Novozyme234 , 6 days culture and 28°C was used for protoplast releasing process. I–I is the standard error bar.

t-test analysis

Data points comparison (*osmotic stabilizer / concentration [M])	t-values, p-values	Conclusions from comparing the means at the 0.05 level.
man/0.8 vs ms/1.0	$5.2234e^{0}$, $6.4125e^{-3}$	Different.
man/0.8 vs man/0.6	$3.5938e^{0}$, $2.2885e^{-2}$	Different.
+man/0.8 vs ms/0.8	$1.9213e^{0}$, $1.2708e^{-1}$	Not different.

P = 0.05 level.

*: man - mannitol solution, ms - magnesium sulfate.

+: data from protoplasts collecting efficiency (figure 3.9.).

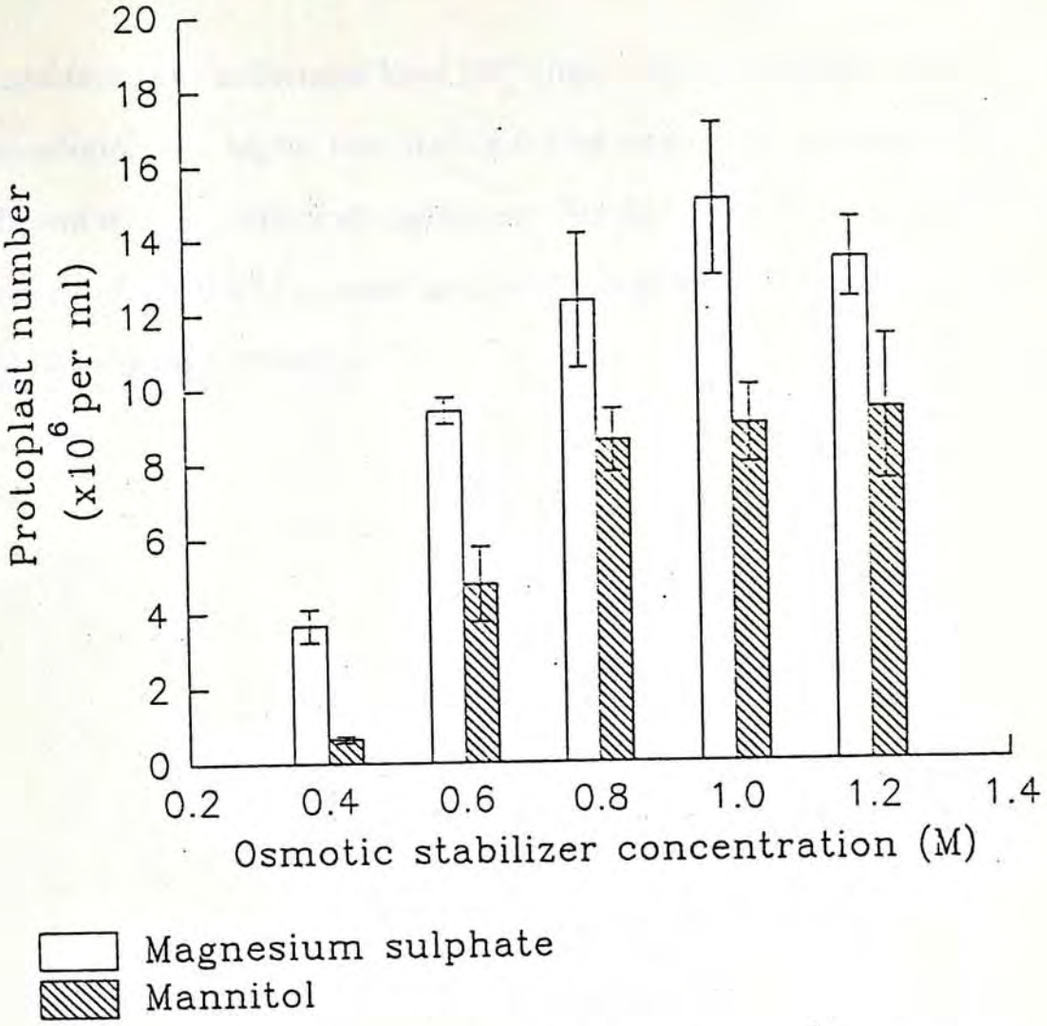


Figure 3.8. Amount of Schizophyllum commune protoplast released by two different osmotic stabilizers in different concentrations. 5mg/ml Novozyme234 and 18mg/ml Lywallzyme, 15 hours culture and 28°C was used in the protoplast releasing process. I–I is the standard error bar.

t-test analysis

Data points comparison (*osmotic stabilizer / concentration [M])	t-values, p-values	Conclusions from comparing the means at the 0.05 level.
man/0.8 vs ms/1.0	$5.2234e^{0}$, $6.4125e^{-3}$	Different.
man/0.8 vs man/0.6	$3.5938e^{0}$, $2.2885e^{-2}$	Different.
+man/0.8 vs ms/0.8	$1.9213e^{0}$, $1.2708e^{-1}$	Not different.

P = 0.05 level.

*: man - mannitol solution, ms - magnesium sulphate.

+: data from protoplasts collecting efficiency (figure 3.9.).

3.3.4. Collecting efficiency of protoplasts by centrifugation

Considering the collectable % of Pf67 (figure 3.9.), the mean value for 0.8 M mannitol solution was higher than that of 0.8 M magnesium sulphate solution but their different was not statistically significant. For Sc17, figure 3.9. showed that the collectable % of the 0.8 M mannitol condition was about five folds greater than that of 0.8 M magnesium sulphate.

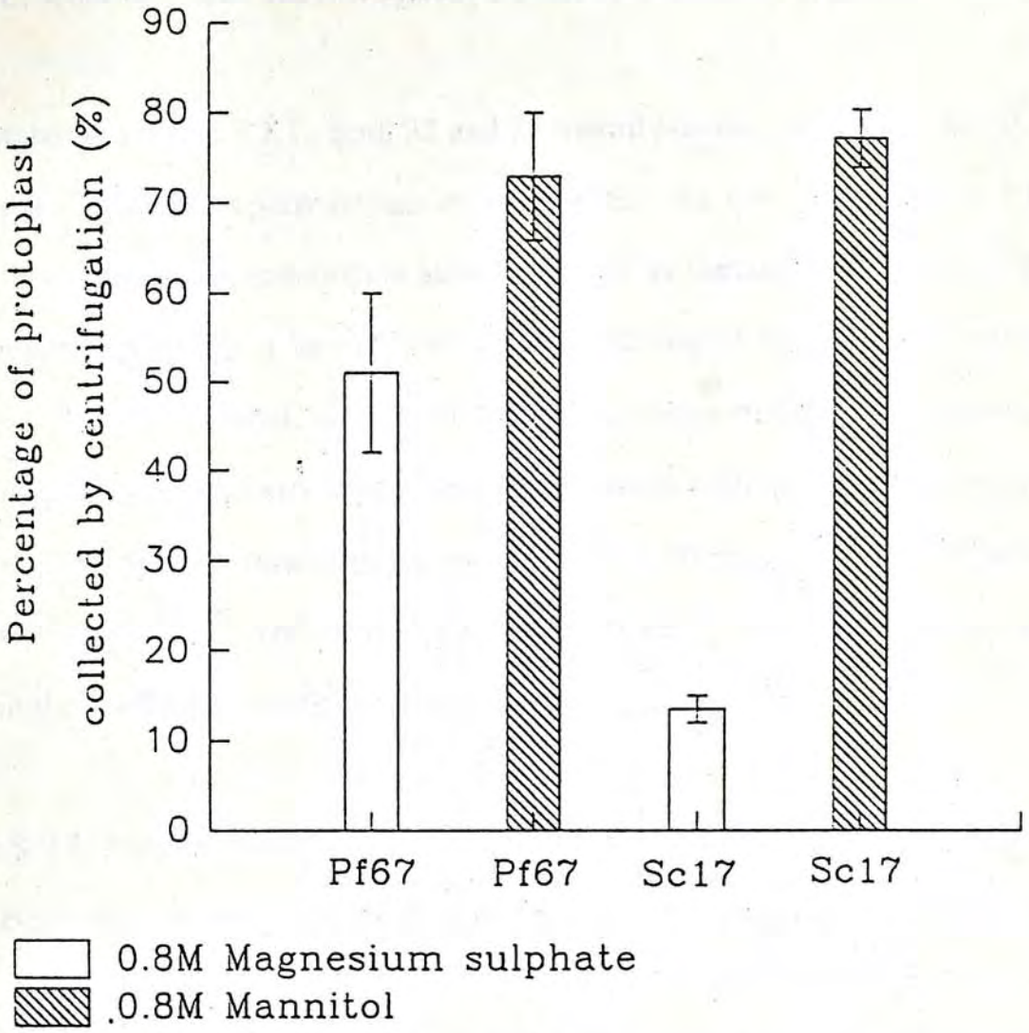


Figure 3.9. The percentage of protoplast collected by centrifugation of Pleurotus florida (Pf67) and Schizophyllum commune (Sc17) in two different osmotic stabilizers : 0.8M Magnesium sulphate and 0.8M Mannitol.

3.4. Discussion

3.4.1. Choice of lytic enzyme system and time for enzyme digestion

As stated in section 3.3.1., both 22 and 24 mg/ml Novozyme234 was found to be the best E.C. for protoplast release of Pf67 within the test series (figure 3.1). However, considering the economical aspects as well as the ability to maintain the high protoplast amount in a longer time interval, 22 mg/ml Novozyme234 at 90 minutes was found to be most suitable for protoplast release of Pf67. The reason is that the 22 mg/ml E.C. showed to be able to maintain the high protoplast amount (4 $\times$ 10^7 protoplast per ml) condition for approximately 30 minutes (from 60 to 90 minutes incubation time). The results of the 24 mg/ml E.C. showed to be less able to maintain the maximum protoplast amount level.

For Sc17, 5 mg/ml Novozyme234 showed to be most effective for protoplast release. However, the maximum amount of protoplasts released in such condition was only about 2.5 $\times$ 10^5 protoplasts per ml at 120 minutes which is rather low. For the purpose of protoplast fusion experiment, the protoplast population of the two strains should be higher (Deed and Seviour, 1990). On the other hand, protoplast yield of 10^8 per ml has been reported for this species (Horton and Raper, 1991). Hence, the second part of the experiment was to improve the protoplast yield of Sc17. Considering the results of figure 3.2, further increase in Novozyme234 to 6 or 7 mg/ml will not improve the protoplast yield. Similar results has also been reported in 1972 by de Vries and Wessels. Therefore, a combined enzyme system for protoplast production was carried out. The results showed that using both Novozyme234 along with Lywallzyme at the tested proportion able to enhanced the protoplast yield of Sc17 approximately five fold,

which is about 5.5×10^6 protoplasts per ml than the additive effect of using the two enzymes independently (figure 3.3.). This experiment verified the potential of improving protoplast yield using the combined enzyme system. Part 3 experiment was then carried out in order to determine the optimum combination ratio of the two enzymes. The results showed that there was an increase in mean protoplast yield when the Lywallzyme concentration increased from 9 to 18 mg/ml (figure 3.4.). Therefore, for the purpose of maximizing protoplast yield, 18 mg/ml Lywallzyme with 5 mg/ml Novozyme234 at 150 minutes was chosen for the optimum enzyme condition for protoplast release of Sc17.

3.4.2. Culture age for maximum protoplast yield

Although the results in figure 3.5. indicated that both 4 days and 6 days Pf67 cultures had similar performance on protoplast yield, the 5 days cultures showed to have the highest protoplast yield. Therefore, 5 days cultures was chosen as the optimized culture age for protoplast isolation of Pf67. However, the optimum culture age of Sc17 for protoplast isolation was found to be much shorter and within 18 hours incubation in 28 °C (figure 3.6.). The highest protoplast yield was obtained from the 15 hours cultures. From the results of the two figures mentioned above, it also showed that culture age is a critical factor in the determination of protoplast yields. The resistance of the mycelium to enzyme degradation increases with culture age. Moreover, the effect of culture age seems to be much more important for protoplast isolation of Sc17. For one thing is that the culture age range with high protoplast yield was found to be only 9 hours and different from that of Pf67 which was about 3 days long. Similar results were obtained by Yanagi *et al.* in 1985. For protoplast isolation of *Pleurotus florida*, they used the 5-day

cultures. For protoplast isolation of *Schizophyllum commune*, Specht *et al.* (1991) also used a 20 hours culture for producing protoplasts.

3.4.3. Choice of concentration and type of osmotic stabilizers

From figure 3.7., 0.8 M mannitol showed to be the most suitable osmotic stabilizer for maximizing the protoplast yield of Pf67. On the aspect of collecting percentage, the results also showed that 0.8 M mannitol was more suitable for protoplasts isolation of Pf67. Considering protoplast isolation of Sc17, although the protoplast yield using mannitol was about 30 % lower than that using magnesium sulphate, the collecting % of protoplast in mannitol was six folds higher than that of magnesium sulphate. Therefore, the optimized choice of concentration and type of osmotic stabilizer for protoplast release of Sc17 should be a compromise of the two factors and should be 0.8 M mannitol which is the same as that of Pf67. Peberdy *et al.* (1976) reported that the concentration of osmotic stabilizer generally varies between 0.4 M and 0.6 M. However, my optimized condition for the two strains was found to be 0.8 M mannitol. Although my result was slightly different from that of Peberdy *et al.* 's, it was not a new founding. Early in 1985, Hou and Jong reported that for sorbitol and mannitol, 0.8 M was the optimum concentration for *Penicillium digitatum* whilst 0.6 M was best for NaCl, KCl, $CaCl_2$ and $MgCl_2$. Moreover, they found that lower concentrations resulted in a loss of intact protoplasts, while higher concentrations inhibited protoplasts release from the mycelium. All these observations was similar to those of my experiments.

From the experiment of protoplasting Sc17 in magnesium sulphate (section 3.2.4.), it was found that there was a difficulty in counting the protoplasts. The reason was that there was only a low proportion of protoplasts able to "sink" onto

the surface of the checker-board of haematocytometer. Most of the Sc17 protoplasts in magnesium sulphate were "floated" onto the bottom surface of the glass cover slide. No study has been made about this aspects before. However, the "floating" phenomenon may related to the buoyancy ability of the protoplasts which in turns affected by the relative concentration of the solute inside the protoplast and the concentration of solute (e.g. osmotic stabilizer) in the bathing solution. As the protoplast membrane is a selectively permeable membrane, change in both passive and metabolically active process(es) may affect the relative solute level of the protoplast cytoplasm. In fact, it is a frequent observation that metabolic activity is affected or impaired in the presence of a particular osmotic stabilizer (Fawcett et al. 1973, Isaac 1985 and Dlugonski et al. 1984). However, most of these studies were based on *Aspergillus nidulans* or *Cephalosporium acremonium*. No direct evidence can be found on this aspect for *Schizophyllum commune*.

In order to remove the cell debris and unlysed mycelial fragments in the protoplast solution, the last step of protoplast preparation was carried out by filtering the protoplast solution through 1 cm thick cotton wool after the protoplast formation procedure described above.

In summary, the optimized conditions for the two strains were as shown in table 3.2.

Table 3.2. Optimized protoplast release condition of Pf67 and Sc17.

	Fungal strains	
	Pf67	Sc17
Culture age	5 days	15 hours
Lytic enzyme : type and concentration	22 mg/ml Novozyme234	5 mg/ml Novozyme234 and 18 mg/ml Lywallzyme
Lytic enzyme digestion duration	90 minutes	150 minutes
Osmotic stabilizer : type and concentration	0.8 M mannitol	0.8 M mannitol

CHAPTER 4

Protoplast fusion of *Pleurotus florida* and *Schizophyllum commune*

4.1. Introduction

This chapter is mainly concerned about the method of protoplast fusion as well as the effects of different experimental steps on various aspects, such as the viability of protoplasts, stability of genetic markers, of the two fusion parents. Both of the physical approach such as electrofusion and the chemical approach such as the polyethylene glycol (PEG) - induced fusion was realized and described by many scientists many years ago (Cocking, 1973, Ferenczy, 1981 and Halfmann et al, 1982.). Although electrofusion has the advantage of high yield of fusion products, it has the main limitation on the scale of fusion experiment (Peberdy, 1987). Moreover, considering both the availability of materials and equipment, PEG-induced fusion was used in this study.

The successful application of protoplasts in fusion experiment clearly depends on the ability of the protoplast to recover to mycelial form after the treatment of fusogen (PEG). On the other hand, the stability of genetic markers through out the fusion process is also a prerequisite for a successful screening process of fusion products in fusion process. Therefore, the viability of protoplast at step 1 and 2 stated in figure 4.1. was studied in this chapter. In

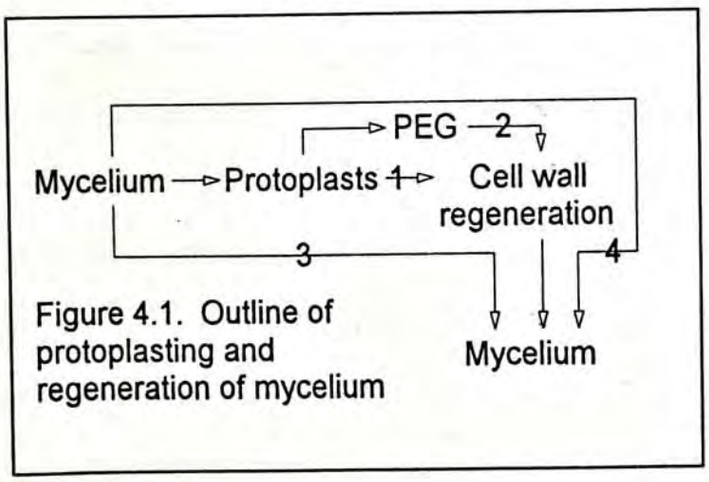


Figure 4.1. Outline of protoplasting and regeneration of mycelium

addition, the back mutation frequency of the genetic markers in experimental route 3 and 4 were also being assessed.

On the cytological aspect of the present protoplast fusion study, one of the objectives was to bring the genomes of the two fusion parents together into the same cytoplasm. However, if large proportion of protoplasts prepared from either fusion parent contained no nucleus, the resulting viable cybrid would have a high chance of carrying only the genome of either parent but not both. Such situation would violate the original purpose of the study. Therefore, fluorescent staining of protoplast's nuclei was carried out in order to determine the proportion of nucleus bearing protoplasts.

4.2. Materials and methods

4.2.1. Protoplast formation and size of protoplasts

The strains used in the protoplast fusion experiment were *Pleurotus florida* Pf67 and *Schizophyllum commune* Sc17. Both strains possess a few genetic markers such as drug resistance markers as well as auxotrophic markers. Details of all these markers of the two strains were described in table 3.1.. The methods for protoplast formation of the two strains were the same as the optimized specific conditions described in table 3.2. of chapter 3. The diameter of fifty protoplasts from the each of the two strains were also measured by ocular micrometer scales along with a 400 × phase contrast light microscope [Zeiss]. Three batchs of protoplasts of each of the two strains were investigated.

4.2.2. Fluorescent staining of protoplasts' nuclei

The nuclei staining method described here was a modified procedure of the one described by Meixner and Bresinsky (1988). Protoplasts of either fusion parents were collected and resuspended in 1 ml dye solution. The dye solution contained 0.8M mannitol as osmotic stabilizer, 0.5 µg DAPI (4',6-diamidino-2-phenylindole) [SIGMA] in McIlvanine's 0.1 M citrate ($C_6H_8O_7.H_2O$) / $Na_2HPO_4 \cdot 2H_2O$ (1 : 5.66) buffer (pH 7.0). Protoplasts were bathed in the dye solution for 15 minutes at 37°C. Then, the stained protoplasts were spun down by centrifugation and resuspended in washing solution twice for 15 and 7 minutes at 37 °C respectively. The washing solution had the same composition as the dye solution but without DAPI. After removing the excessive DAPI from the protoplasts by washing solution, protoplasts were mounted onto glass slide with the washing solution. For fluorescence microscopic observations, the slide was observed using a Nikon Biophot [Nikon] epifluorescent microscope equipped with 100-W mercury vapor lamp. Observations were made by using filter combination UV-1A, giving a peak of excitation light between 330 nm and 380 nm. For each of Pf67 and Sc17 protoplasts, the number of nuclei in one hundred protoplasts were recorded. Totally one hundred protoplasts were observed. That is, protoplasts inside each view of microscope were observed and counted under bright field and then the number of protoplasts bearing different numbers of nuclei in the same view were observed and counted under UV illumination.

4.2.3. Stability of the genetic markers

4.2.3.1.Preparation of media for checking the presence of genetic markers

The stability of the genetic markers of the two fusion strains throughout the procedures of fusion experiment were assessed. Different media were used for checking the markers.

For drug resistance markers, complete medium (CM) was prepared with the composition as follows : 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.46 g/L KH_2PO_4, 1.0 g/L K_2HPO_4, 2.0 g/L Bacto-peptone [DIFCO], 2.0 g/L Bacto-yeast extract [DIFCO], 20 g/L glucose [BIOLIFE], 50 mg/L Thiamin-HCl [SIGMA] and 20 g/L Bacto-agar. For checking the acriflavin resistance character, 100 mg/ml acriflavin [SIGMA] stock solution was prepared by dissolving acriflavin in distilled water. The stock solution was then sterilized by filtration through Schleicher & Schull membranfilter. The pore size of the membranfilter was 0.2 μm. One milliliter of acriflavin stock solution was added to 1 L of molten CM at 50 ^oC. The final concentration of the acriflavin in CM was 100 μg/ml (CM_{Acr}). For determining guaiacol resistance marker, 1.11 mg/ml guaiacol [SIGMA] stock solution was prepared instead. One milliliter of this stock solution was added to the 1 L CM to produce a guaiacol medium with final concentration of 1 μg/ml guaiacol (CM_{Gua}).

Minimal medium (MM) was used for checking the auxotrophic nature of the strains. It was composed of 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.46 g/L KH_2PO_4, 1.0 g/L K_2HPO_4, 0.12 g/L DL-asparagine, 20 g/L glucose, 120 mg/L Thiamin-HCl and 20 g/L Bacto-agar (Raper and Raper, 1972). Supplemented medium was used for clarification of the type of the auxotrophic character of the strains. Adenine [SIGMA] and nicotine [SIGMA] stock solutions were prepared by dissolving 5 mg of either amino acid into 100 ml distilled water. The solutions were sterilized by millipore similar to the preparation of acriflavin stock solution described above. One milliliter of the amino acid's stock solution was added into 100 ml molten minimal

medium at 50 °C. Therefore, the final concentration of each amino acid in the supplemented minimal medium was 0.5 μg/ml. By the above method, MM supplemented with adenine (MM_{ade}), MM supplemented with nicotine (MM_{nic}) and MM supplemented with both adenine as well as nicotine ($MM_{ade,nic}$) was prepared.

4.2.3.2.Determining the presence of auxotrophic as well as drug resistance markers

Culture of the fungal strain was maintained on PDA medium. One thin mycelial disc , with a dimension of 2 mm diameter and about 1 mm thick, was picked from the culture on PDA and inoculated onto each of the following testing media, i.e., MM, CM, CM_{Acr}, CM_{Gua}, MM_{ade}, MM_{nic} and $MM_{ade,nic}$. These testing culture plates were then incubated at 28 °C for 7 days in darkness. After incubation, the inoculated mycelia on all testing culture plates were being inspected and recorded for the presence of mycelial growth.

Another similar experiment was also carried out. Twenty colonies of Pf67, which were regenerated from the protoplasts on the RCM plates after PEG treatment (figure 4.2.), were subculture onto PDA. Another twenty regenerated colonies of Sc17 were also collected in a similar way as that of Pf67. The presence of auxotrophic as well as drug resistance markers in these, totally forty, subcultures were checked in the same way as those without PEG treatment. The whole experiment was then repeated. The total number of colonies showed growth on the test medium was recorded.

4.2.4. Regeneration of mycelium from protoplasts

Regeneration of mycelium from protoplasts was carried out on regeneration medium. There are two types of regeneration media. They are the regeneration complete medium (RCM) and the regeneration minimal medium (RMM). These two regeneration media had the basic components similar to that of CM and MM respectively. However, in the preparation of these two media, 0.8 M of mannitol was added to each media. The concentration of the mannitol was the same as the result of optimal osmotic stabilizer's condition described in section 3.4.3..

After protoplasts were released under the conditions discribed in table 3.2., the concentration of protoplasts was counted (totally 2 ml, and generally had a protoplasts amount approximately equal to 10^7 protoplasts per ml). For the studying the BMF of the fungal strain, one microliter of this protoplast solution was spread onto five RMM plates in 200 ul aliquot portions. For the RF studies, the remaining protoplast solution was diluted by 0.8 M mannitol solution to 10^5 protoplasts per ml. 200 μl of the diluted protoplast solution were then spread onto each of the five RCM plates. The spread plates were then incubated at 28 ^{o}C. Three days incubation were needed for the regeneration of Sc17 protoplast into observable (naked eyes) colonies. However, 5 days were needed for the regeneration of Pf67 protoplast. The regeneration frequency (RF) as well as the back mutation frequency (BMF) studied was defined as follows:

$$\text{Regeneration frequency (RF)} = \frac{\text{Number of regenerated colonies on the RCM regenerating medium plate}}{\text{Number of protoplasts spread on the RCM regenerating medium plate}} \times 100\ \%$$

$$\text{Back mutation frequency (BMF)} = \frac{\text{Number of regenerated colonies on the RMM regenerating medium plate}}{\text{Number of protoplasts spread on the RMM regenerating medium plate}} \times 100\ \%$$

For investigating the effect of different steps in the fusion experiment on the regeneration ability of the protoplasts, both RF as well as BMF before and after the polyethylene glycol (PEG) treatment were determined. Moreover, a water-lysed experiment was also carried out to determine the amount of mycelial fragments remained in the protoplast solution after protoplast preparation. Procedures were similar to those for determining RF or BMF except that sterilized distilled water was used instead for diluting the protoplast solution. When the protoplast solution was diluted 10 to 100 folds with sterilized distilled water instead of 0.8 M mannitol solution (in the RF experiment, dilution was necessary to attain a protoplast solution with 10^5 protoplast per milliliter concentration), the concentration of osmotic stabilizer was decreased to 0.08 to 0.008 M. This concentration of osmotic stabilizer could no longer maintain the osmotic balance of the protoplasts and cell bursting was resulted. For complete elimination of the possibility of protoplast regeneration, the water lysed protoplast solution was spread on CM instead of RCM (figure 4.2.). As mycelial cell had cell wall and will not be burst, hence, any colony regenerated after spreading and incubation was originated from the mycelial fragment of the protoplast solution .

In addition, the morphological forms of regeneration of mycelia from protoplast of the two strains on RCM were also being inspected by using a 400 × phase-contrast light microscope [Zeiss]. The regeneration forms of 50 protoplasts of each of Pf67 and Sc17 were recorded. The above microscopic observation experiments were then repeated in another batch of experiment. Results were

recorded as the mean percentage of occurrence. t-test analysis (independent) at P = 0.05 level was then carried out for statistical comparison of the occurrence of the three regeneration types.

4.2.5. Protoplast fusion and screening of fusion products

The simplified experimental outline of the fusion experiment was shown in figure 4.2.. Fusion of protoplasts was carried out by a chemical fusogen, i.e., polyethylene glycol (PEG). Protoplast fusion was performed according to the method described by Anne and Peberdy (1976).

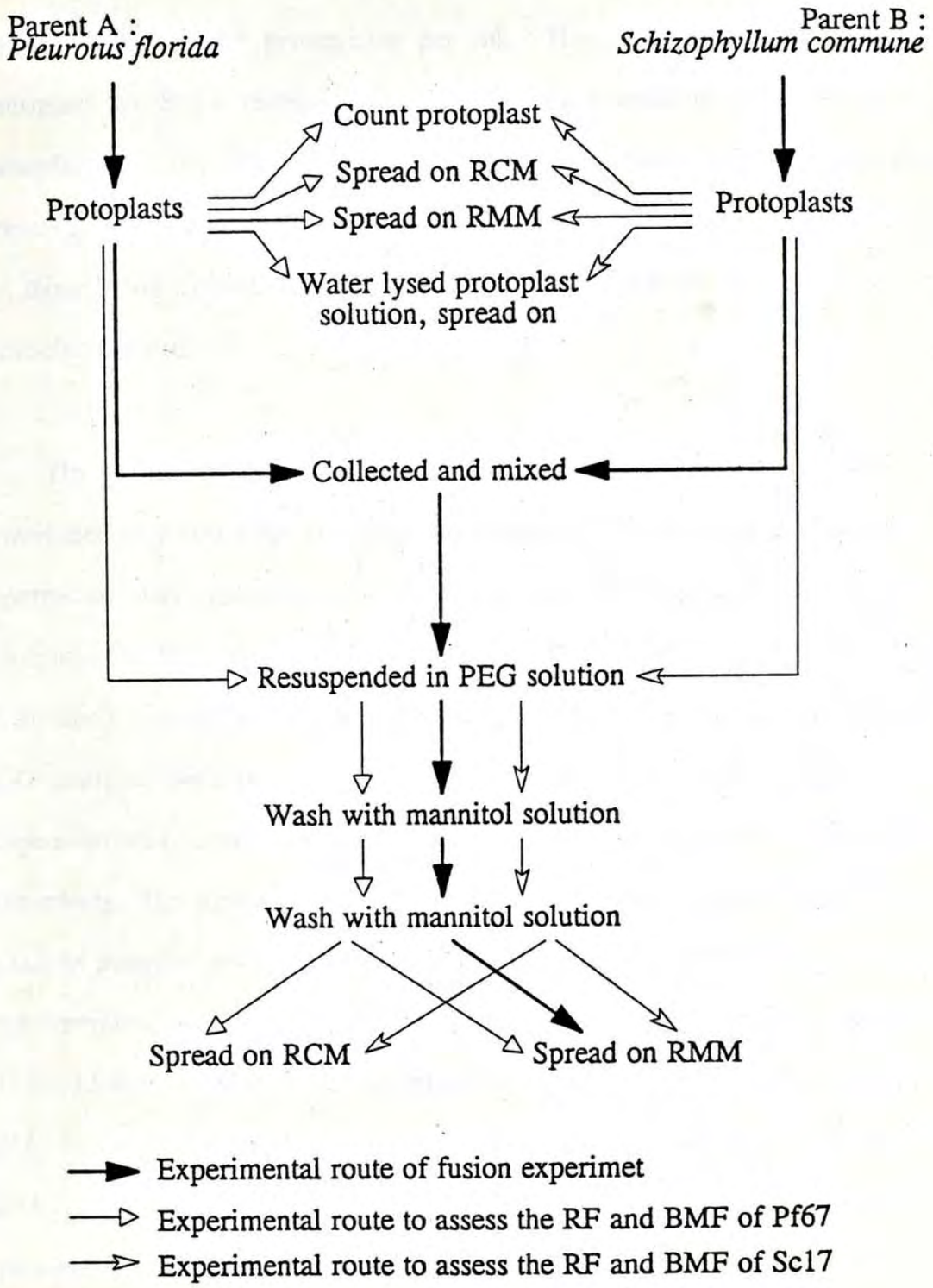


Figure 4.2. Outline of Protoplast Fusion Experiment

After protoplasts were released by the method described in section 3.4., 1 ml protoplast solution was obtained from each parents. As indicated from the results of section 3.3, the amount of protoplasts released from Pf67 under optimized conditions was about 40×10^6 protoplasts per ml. However, the maximum number of protoplast of Sc17 released under optimized conditions was about 9×10^6 protoplasts per ml. In order to attain an approximately 1 : 1 ratio of protoplast amount of the two strains for fusion, the protoplast amount of each of the two strains for mixing and collecting by centrifugation was kept to approximately 1×10^7 protoplasts per ml.

The protoplast solution with 10^7 of Pf67 and 10^7 of Sc17 protoplasts was then centrifuged at 1,400 g for 10 minutes by Hettich EBH 3S table top centrifuge. The supernatant was discarded and the protoplast pellet was resuspended in PEG solution. The PEG solution contained 30% w/v of polyethylene glycol 4,000, 0.05 M glycine / sodium hydroxide buffer of pH 7.5 and 0.05 M calcium chloride. The PEG solution with protoplasts was incubated at 28 ^{o}C for 15 minutes. The suspension was centrifuge at 1,400 as discribed above in order to spin down the protoplasts. The supernatant was discarded. The pellet was washed by resuspending in 0.8 M mannitol twice. Then, each of 0.2 ml aliquots of the resuspended solution was separately spread into RMM. The spread RMM plates was then incubated at 28 ^{o}C for 21 days. Colony(ies) regenerated from the RMM plates was subculture into MM plate and incubated at 28 ^{o}C again. Until mycelia was grown onto the MM, the newly grown mycelia was subculture and maintained on PDA medium for further analysis.

4.3. Results

4.3.1. Size of protoplasts of Pf67 and Sc17

As the smallest unit of the ocular micrometer is 2.5×10^{-3} mm, the diameter of protoplasts was expressed as multiple of this dimension (figure 4.3.). For Pf67, the diameters of protoplasts fell within the range of 2.5×10^{-3} mm to 7.5×10^{-3} mm . The protoplasts' diameters of Sc17 fell within the range of 2.5×10^{-3} mm to 15×10^{-3} mm. By t-test analysis at $P = 0.05$ level, most of the Pf67 protoplasts were 2.5 to 5.0×10^{-3} mm in diameter. For Sc17, most of the protoplasts had their diameters of 5.0 to 7.5×10^{-3} mm. These two groups of protoplasts had their frequencies significantly higher than that with other diameters.

4.3.2. Proportion of protoplasts bearing nuclei

The results showed that DAPI was an effective nuclear fluorescence stain for both Sc17 (figure 4.4.) and Pf67 (figure 4.5.). The results on the proportion of protoplast bearing nucleus were recorded in table 4.1..

Table 4.1. Percentage of protoplast bearing different number of nucleus

	Percentage of protoplasts bearing			
	no nucleus	one nucleus	two nuclei	more than two nuclei
Sc17	10	78	12	0
Pf67	35	58	7	0

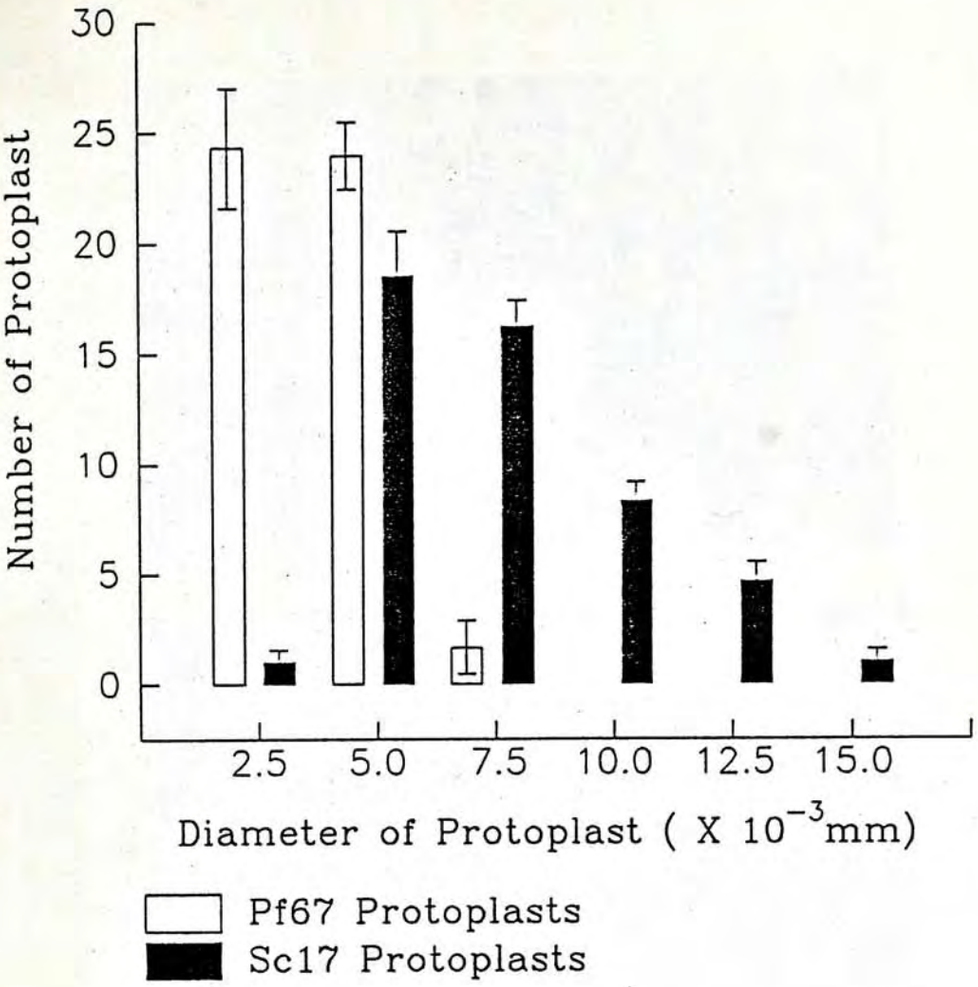


Figure 4.3. The size of protoplasts of Pf67 as well as Sc17 released under the optimal condition determined in chapter 3. I–I is the standard error bar.

t-test analysis

Data points comparison (Strain / diameter [mm])	t-values, p-values	Conclusion from comparing the means at the 0.05 level.
Sc17/5.0 vs Sc17/7.5	$9.8995e^{-1}$, $3.7823e^{-1}$	Not different
Sc17/7.5 vs Sc17/10.0	$5.3666e^{0}$, $5.8207e^{-3}$	Different
Pf67/2.5 vs Pf67/5.0	$1.0660e^{-1}$, $9.2024e^{-1}$	Not different
Pf67/5.0 vs Pf67/7.5	$1.1490e^{+1}$, $3.2749e^{-4}$	Different

P = 0.05 level.

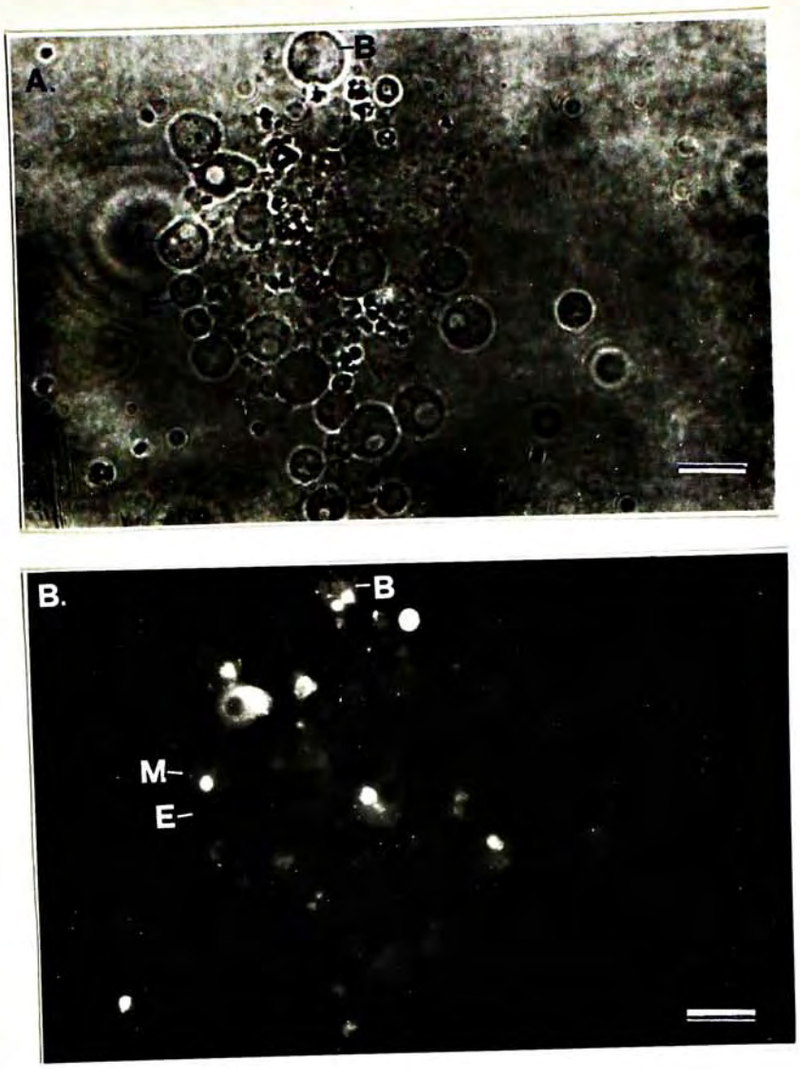


Figure 4.4. Protoplasts of *Schizophyllum commune* (Sc17). (A) Light micrograph of Sc17 protoplasts. (B) Fluorescent micrograph showing nucleus of protoplasts stained by DAPI. M, mononucleate protoplast; B; binucleate protoplast; E, protoplasts without nucleus. Both micrograph (A) and (B) were taken under the same view (scale bar, 10 μm).

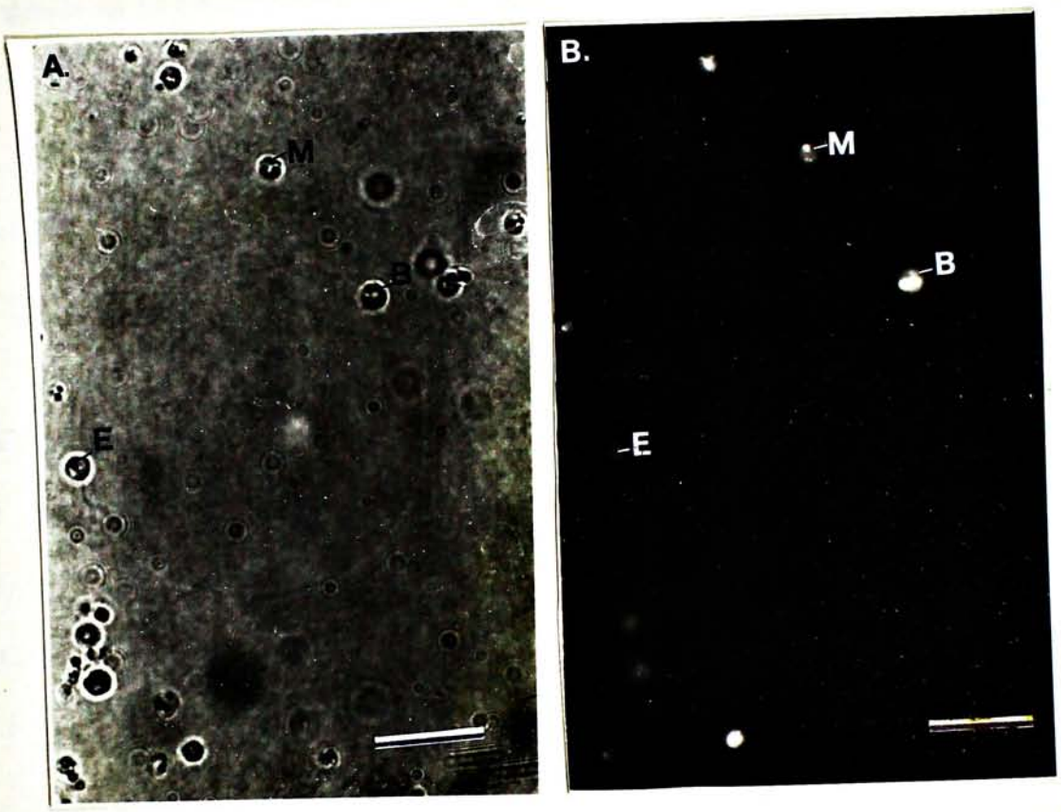


Figure 4.5. Protoplasts of *Pleurotus florida* (Pf67). (A) Light micrograph of Pf67 protoplasts. (B) Fluorescent micrograph showing nucleus of protoplasts stained by DAPI. M, mononucleate protoplast; B, binucleate protoplast; E, protoplasts without nucleus. Both micrograph (A) and (B) were taken under the same view (scale bar, 10 μm).

4.3.3. Protoplast regeneration in regeneration medium

4.3.3.1.Protoplasts regeneration morphologies

Based on the morphologies observed by microscopy, three regeneration forms were found : type I - 'bud and hyphae', type II - 'segmented hyphae' and type III - 'direct hyphae'. Protoplasts with type I regeneration form showed to developed a bud-like structure in between the emerged hyphae and the protoplast. Protoplasts with type II regeneration able to generate mycelial hyphae from the protoplast, however, the regenerated hyphae showed to be segmented rather than a long and smooth tube like hyphae. Type III regeneration form reflected the emerging of a long and smooth tube like hyphae form the protoplast.

Both type I (figure 4.6.A.) and type III (figure 4.6.B.) regeneration forms were observed in Sc17 protoplasts. Pf67 protoplasts showed all three types of regeneration forms (figure 4.7.A and 4.7.B). However, type I and type II regeneration forms of Pf67 protoplasts were not mutually exclusive to each other (figure 4.8.).

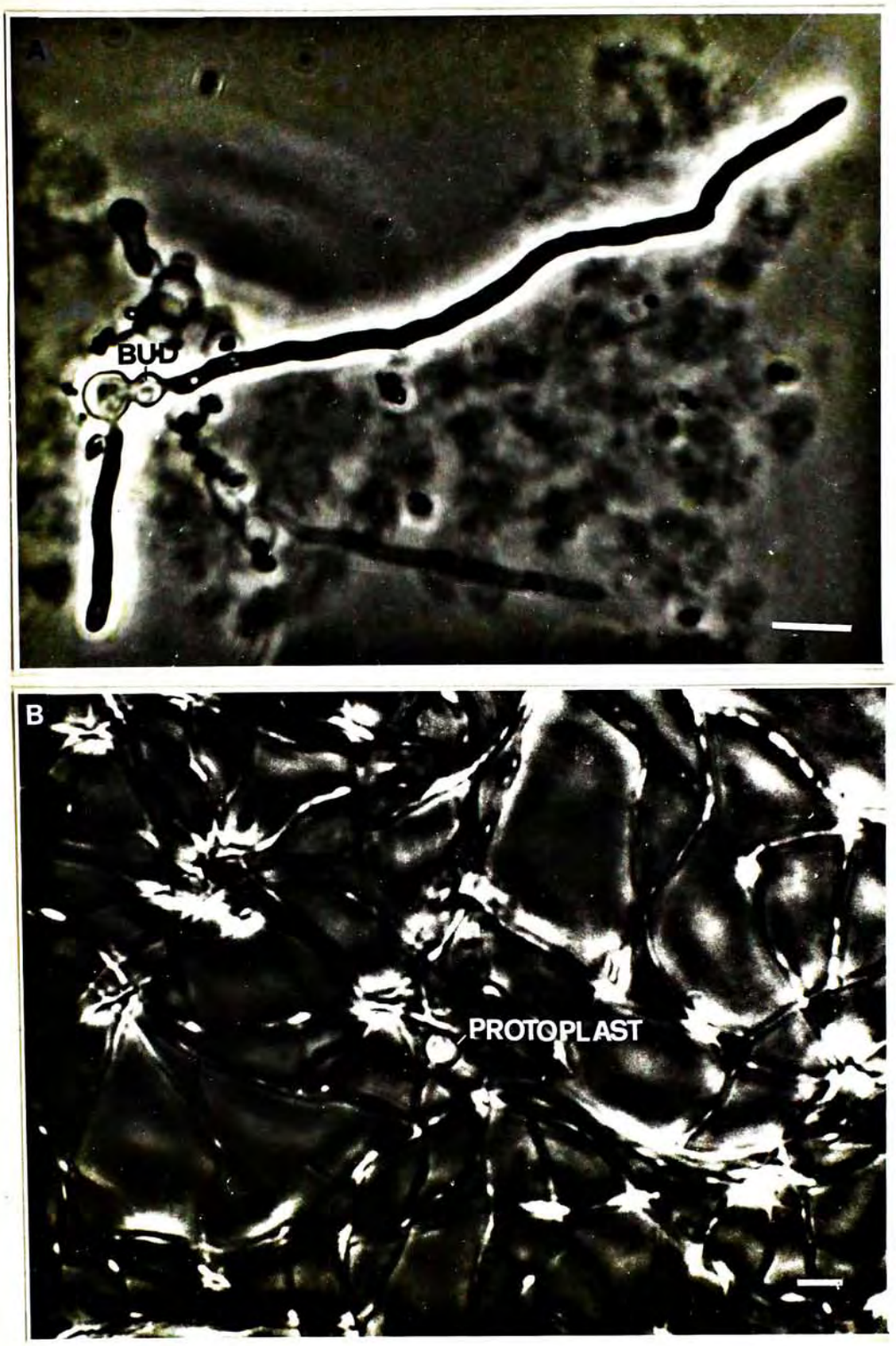


Figure 4.6. Light micrographs showing different regeneration morphologies of *Schizophyllum commune* (Sc17) protoplasts. (A) Type I regeneration of protoplast which shows a bud-like morphology in between the protoplast and the regenerated mycelium. (B) Type III regeneration of protoplast which shows a direct emergent of mycelium form the protoplast (scale bar, 10 μm).

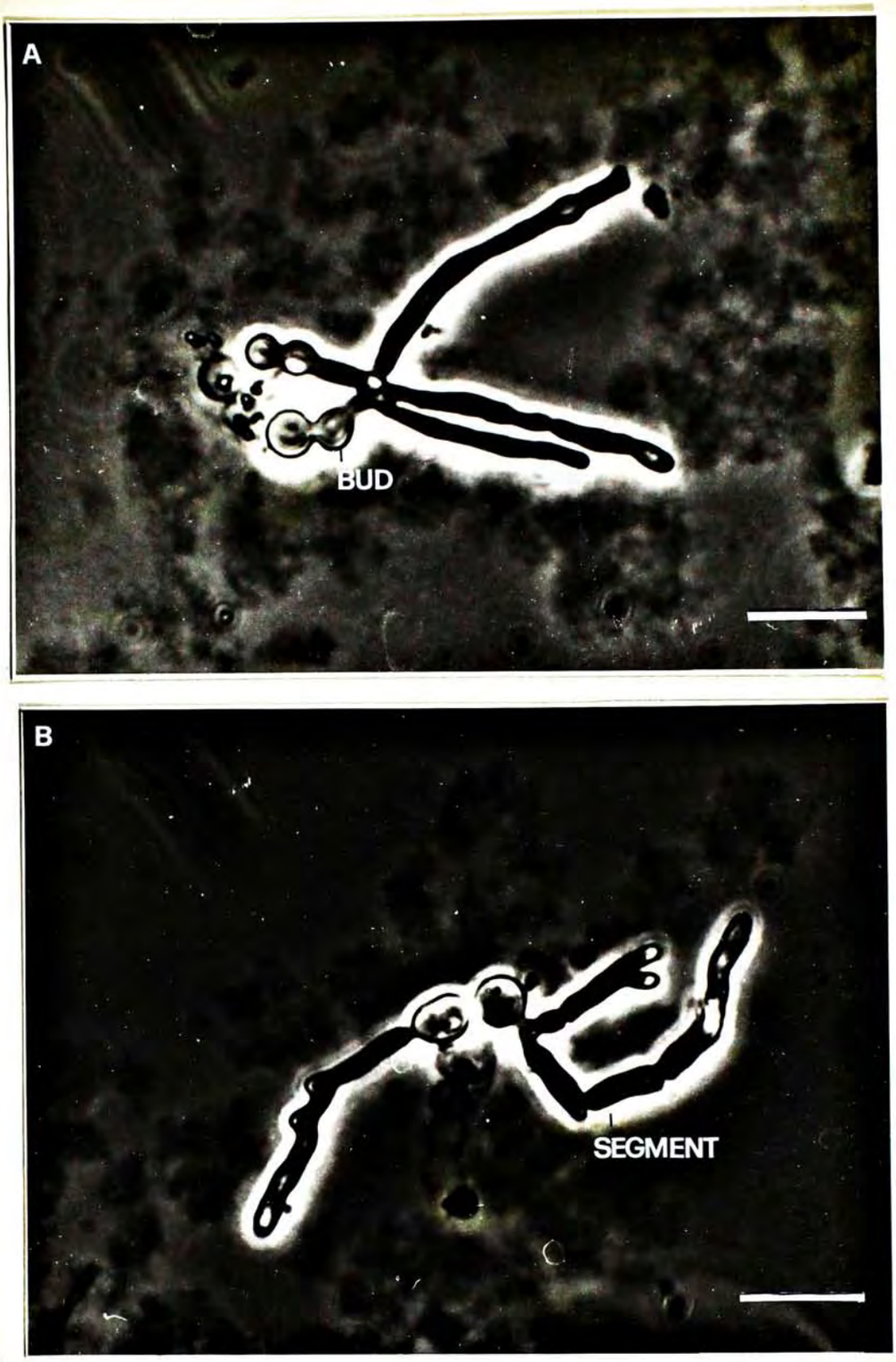


Figure 4.7. Light micrographs showing different regeneration morphologies of *Pleurotus florida* (Pf67) protoplasts. (A) Type I regeneration of protoplast which shows a bud-like morphology in between the protoplast and the regenerated mycelium. (B) Type II (2) and type III (3) regeneration of protoplast which shows a direct emergent of mycelium form the protoplast and formation of segment like mycelium form protoplast (scale bar, 10 μm).

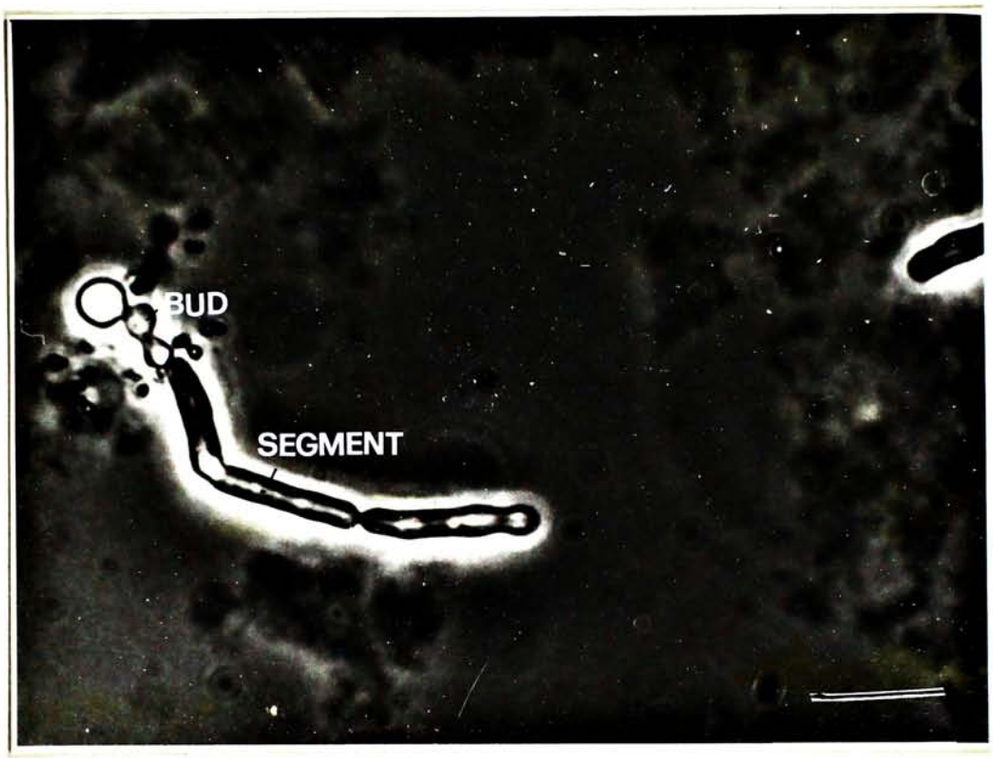


Figure 4.8. Light micrograph showing a single regenerating protoplast of *Pleurotus florida* (Pf67) with both type I (1) and type II (2) regeneration morphologies (scale bar, 10 μm).

The percentages of different regeneration type for the two strains was shown in table 4.2. and the results of t-test analysis (independent) was shown in table 4.3..

The results of t-test showed that both Pf67 and Sc17 protoplasts to have the highest proportion of type III regenerating form.

Table 4.2. The percentage of different regeneration forms for Pf67 and Sc17 protoplasts.

Regeneration forms	Pf67	Sc17
Type I	15 %	41 %
Type II	17 %	Nil
Type III	67 %	59 %
Mixed type (I and II)	1 %	Nil

Table 4.3. t-test analysis (independent) of the percentages of different regeneration forms.

	Pf67			Sc17
Comparing groups	type III vs I	type III vs II	type II vs I	type I vs III
t-values, P-values	1.4145e+1, 4.9609e-3	1.5811e+1, 3.9167e-3	4.4721e-1, 6.9848e-1	1.2728e+1, 6.1163e-3
Statistical differences	Different	Different	Not different	Different

4.3.3.2.Regeneration frequencies and back mutation frequencies of Pf67 and Sc17 protoplasts

The RF and BMF of Pf67 as well as Sc17 protoplasts before and after PEG treatment was summarized in table 4.4.. The statistical comparison of the RF values was shown in table 4.5..

Table 4.4. Mean RF and BMF of Pf67 and Sc17 protoplasts of three fusion experiments.

	RF			BMF	
	Before PEG treatment (%)	After PEG treatment (%)	Water-lysed control (%)	Before PEG treatment (%)	After PEG treatment (%)
Sc17	0.4741	0.4190	7.2472×10^{-5}	N.D.*	N.D.
Pf67	0.6466	0.6103	1.7915×10^{-5}	N.D.	N.D.

*Remark : N.D. - Not detectable, therefore, less than 10^{-5} %.

Table 4.5. t-test analysis (independent) of the RF of Pf67 and Sc17 protoplasts.

	Pf67	Sc17	Before PEG treatment	After PEG treatment
Comparing groups	Before vs after PEG treatment	Before vs after PEG treatment	Pf67 vs Sc17	Pf67 vs Sc17
t-values, P-values	1.6081e0, 1.8308e-1	1.4040e0, 2.3299e-1	3.9573e0, 1.6715e-2	7.3202e0, 1.8529e-3
Statistical differences	Not different	Not different	Different	Different

4.3.4. Effect of PEG fusion treatment on auxotrophic and drug resistance markers of Pf67 and Sc17

The auxotrophic and drug resistance characters of Pf67 and Sc17 before and after PEG fusion treatment was summarized in table 4.6..

Table 4.6. The viability of Pf67 and Sc17 mycelium in different media.

	Before PEG fusion treatment						
	CM	CM_{Acr}	CM_{Gua}	MM	MM_{ade}	MM_{nic}	$MM_{ade,nic}$
Pf67	+	-	-	-	+	-	+
Sc17	+	+	+	-	-	-	+
	Regenerated after PEG fusion treatment* (number of colonies showed growth)						
	CM	CM_{Acr}	CM_{Gua}	MM	MM_{ade}	MM_{nic}	$MM_{ade,nic}$
Pf67	40	0	0	0	40	0	40
Sc17	40	40	40	0	0	0	40

*The recorded data are the summation of the results of two separated experiments.

4.3.5. Fusion products obtained from screening process

Ten fusion experiments were carried out. Three fusion products, PS1, PS2 and PS3, were obtained separately from the RMM plates of three experiments (figure 4.9.). These fusion products were named according to the first letter of the fusion parents' generic names as well as their regeneration sequence.

Figure 4.9. Regeneration of protoplasts after PEG fusion treatment on regenerating minimal medium (RMM). Mycelia growth from the aggregated mass of protoplasts (scale bar, 100 μm).

4.4. Discussion

4.4.1. Effect of protoplast isolation and PEG treatment on the two fusion parents

As stated in the introduction of this chapter, the protoplast regeneration frequency as well as the stability of genetic markers of the two fusion parents before and after PEG treatment is important for the qualitative assessment of the whole fusion process. On the other hand, besides the effects come from cytological and physiological differences as well as the genetical incompatibility between the fusion parents, successful protoplast fusion experiment generally should also depend on an efficient protoplast isolation process which is capable to produce the maximum number of collectable and regenerable protoplasts (Necas and Svoboda, 1985). In addition, the effect of PEG treatment process on the regeneration ability of the isolated protoplast should be little. Moreover, the effects of these two processes on the genetic markers of the fusion parents should also be a minimum.

From the results showed in table 4.4 and 4.5., the RF of both Sc17 and Pf67 was rather low compared to the results of previous reports. RF values ranged from 7.3 % to 23.8 % has also been reported for *Pleurotus salmoneostramineus* protoplast (Iijima, *et al.*, 1991). However, RF of *Pleurotus ostreatus* was reported to be 0.16 % (Magae *et al.*, 1985) which was a result comparable to that of the present study on *Pleurotus florida* (0.65 %). Magae *et al.* explained the low frequency of regeneration by the large number of enucleate protoplasts (36 %). On the other hand, Peberdy in 1979 also reported that the proportion of enucleate protoplasts of *Pleurotus florida* was 20 % to 50 % (Peberdy, 1979b). Similar result was found in the presence study (35 %). The RF of Sc17 and Pf67 was about 0.47

and 0.65 % respectively. For *Schizophyllum commune*, high RF of 50 % has been reported (De Vries and Wessels, 1975) but the RF of Sc17 found in this study was only 0.65 %. Such large discrepancy seems to be not contributed form the proportion of enucleate protoplast as only 10 % protoplasts were found to lack a nucleus. For both Sc17 and Pf 67, the low RF values may be due to a number of factors other than the proportion of enucleate protoplasts. Several studies reported the low regenerative capacity of *Corprinus macrorhizus* and *Laccaria bicolor* protoplasts isolated by enzymatic activity of Novozyme234 (Yanagi *et al.*, 1985, Kropp and Fortin, 1986). It was possible that this was caused by its high protease activity. Kitamoto *et al.* (1988) reported that *Trichoderma* enzymes showing reduced proteinase activity after treatment with bentonite increased regenerative capacity by up to 1.8 times. Moreover, the low RF of Sc17 and Pf67 may also be explained simply by the short incubation time for protoplast regeneration. The regeneration time used in the experiment were three and five days for Sc17 and Pf67 respectively. However, the regeneration time allowed for counting all colonies in the RF studies of *Pleurotus ostreatus* was 16 to 17 days, although six to seven days incubation could give visible regenerated colonies (Magae *et al.*, 1985). Nevertheless, the exact reason for explaining such large discrepancy found in this study is still unknown.

On the aspect of the stability of genetic markers, the results showed that both the auxotrophic and drug resistance markers were not affected by the PEG fusion process (table 4.6.). Therefore, all these markers showed to meet the criteria as genetic marker for the protoplast fusion experiment.

4.4.2. Structural heterogeneity of protoplasts

Structural heterogeneity of protoplasts released from the two strains were observed in the results of the experiments of section 4.2.2. and 4.2.4.. Such heterogeneity was found to be relatively more intensive in Sc17 than Pf67.

The results of figure 4.3. showed that Sc17 protoplasts had the size (diameter) range spanned from the smallest of 2.5×10^{-3} mm to the largest of 15×10^{-3} mm. However, the Pf67 protoplasts had a relatively more narrow size range of 2.5 and 5.0 $\times 10^{-3}$ mm. Therefore it is reasonable to assume that, for a defined cytoplasmic volume, protoplast isolation of Sc17 should be expected to give lesser number of protoplasts than Pf67. Actually, the results in chapter 3 (figure 3.1. and figure 3.4.) showed that protoplast isolation efficiency (based on the number of protoplasts released only) of Pf67 was found to be approximately seven times greater than that of Sc17.

The results mentioned in the above paragraph, the generally smaller protoplast size of Pf67 than that of Sc17 as well as the higher protoplast isolation efficiency of the former strain, suggested two possibilities about the protoplast isolation mechanism of the two strains. The first possibility was that there may be more than one protoplast generated from each mycelial cell of Pf67 and each Sc17 mycelial cells should be able to generate approximately one protoplasts. The second possibility is that each protoplast of Pf67 should generally be isolated from single mycelial cell and each protoplast of Sc17 should be originated from more than one mycelial cells. Nevertheless, the results of section 4.3.2. showed the first possibility seems to be more reasonable. The reason was that about 90 % of Sc17 possess one or more nuclei. For Pf67 protoplasts, the corresponding result was 65 %. The high proportion of nucleus lacking Pf67 protoplasts should only be explained by the fact that more than one protoplast was produced from each mycelial cell. Similarly, the

80 % of the mononucleate Sc17 protoplast also implied the fact that single protoplast was generally produced from each mycelial cell. However, I would like to point out that the above interpretation was just a generalized picture. Therefore, we should not surprised when we observed the 10 % eunucleated Sc17 protoplasts or the 7 % binucleate Pf67 protoplasts.

The above discussions only proposed and described a possible way of manifestation of the structural heterogeneity of protoplasts of the two fungal strains. Actually, some scientists have already given some suggestions on the reasons for such heterogeneity. Some scientists reported that there might be a hyphal physiological gradient along the fungal mycelium (Gibson and Peberdy, 1972; De Vries and Wessels, 1975). Therefore, an uneven distribution of hyphal content among the protoplast released form different part of the mycelium would happen. Moreover, protoplast release were generally occurred by extruding cytoplasm through weaker points in the cell wall (Peberdy, 1971; De Vries and Wessels, 1972). Therefore, the latter evidence suggested that total wall degradation was not necessary and protoplast formation might occur wherever there was a weaker point along the mycelium cell wall. In addition, the hyphal physiological gradient along the fungal mycelium would give rise to different extent of cytoplasmic extrusion and hence resulting in structural hetergeneity of protoplasts.

Although a certain extent of structural heterogeneity among the protoplasts and the polymorphism among the early stage of protoplast regeneration of the two strains were observed, the genetic markers of the regenerable protoplasts showed to be stable (section 4.3.3.1.). Therefore, all the information described in these two sections showed that the effect of the structural heterogeneity of protoplasts might contribute to the morphological and cytological variation among the protoplasts of

the two strains. However, such kinds of variations did not affect the regenerable protoplasts genetically considering on the aspects of the stability of genetic markers.

4.4.3. Polymorphic nature of protoplast regeneration

The results described in section 4.3.3.1. showed that the regenerants of both Pf67 and Sc17 had a polymorphic nature in the early stage of regeneration. The type I and type III regeneration forms were commonly found for both of the two strains. Actually, such form of protoplast regeneration have been described in the regeneration of protoplasts of *Saccharomyces cerevisiae* (Necas, 1971), *Aspergillus nidulans* (Peberdy and Gilbson, 1971), *Schizophyllum commune* (De Vries and Wessels, 1972), *Pyricularia oryzae* (Kobayashi et al., 1985), *Volvariella volvacea* (Mukerjee and Sengupta, 1986) and *Pleurotus sajor-caju* (Liang and Chang, 1989). For understanding the wall formation mechanisms in these regenerating types, a lot of studies about wall formation of the 'bud-like' Sc17 regenerants have been reported. By using N-acetyl^{3}H-glucosamine for incorporating into cell wall during wall formation, the results of radiography study suggested that budding protoplasts were unable to undergo controlled extension (Gaugy and Fevre, 1985).

4.4.4. Protoplast fusion frequency

In the present study, three fusion products was obtained. Each of them was obtained from separate fusion experiment. Therefore, the fusion frequency was approximately equal to 10^{-5} % considering the successful fusion experiment independently. Such a fusion frequency was very low and comparable to spontaneous mutation rate. Therefore it was reasonable to suspect that the fusion products, in fact, were resulted from back mutation of auxotrophic markers. As

stated in table 3.1. of chapter 3, Sc17 possess two auxotrophic markers, which were adenine and nicotine requiring markers. The back mutation rate of each auxotrophic markers should be equal to the spontaneous mutation rate, that is, 10^{-6}. Thus, the probability of back mutation of both markers to form a prototroph should be 10^{-12}. Hence, if a fusion product which was morphologically Sc17 like and originated from back mutation of the double auxotrophic markers, the probability of occurrence should be 10^{-12} but not 10^{-7} as PS1 and PS2. Therefore, it was unlikely that the PS1 and PS2 were obtained from the results of back mutation. However, as Pf67 only possess one auxotrophic marker, we could not eliminate the probability that the Pf67-like fusion product, PS3, was arose from back mutation of auxotrophic marker. Nevertheless, the physiological, cytological and genetical studies in part III of this thesis would further characterize the three fusion product of obtained from the present fusion system.

On the aspect of the fusion frequency of this protoplast fusion system, the low fusion frequency was not unexpected. Similar intergeneric crosses has also been carried out with the yeast *Candida tropicalis* and *Saccharomycopsis fibuligera* and the fusion frequency was 10^{-5}. Intergeneric hybridization between *Aspergillus niger* and *Trichoderma viride* was also found to have the fusion frequency of 10^{-4}-10^{-5}. However, the 10^{-7} fusion frequency found in the present fusion system was approximately 100 times lower than the about two cases. The exact reason for such a discrepancy was unclear. Actually, any factor that was able to affect the formation of hybrid, such as the techniques in the procedure of fusion experiment, the extend of genetic or chromosomal homology of the fusion parents (Peberdy, 1979a) as well as the screening process, might contribute to the resulting low fusion frequency.

Part III

Analysis of Fusion Parents and Fusion Products

Chapter 5

Morphological and Cytological Studies

5.1. Introduction

In various protoplast fusion experiments, morphological and cytological comparisons between the fusion parents and the regenerants from fusion experiments were some of the basic and common approaches in characterization of the fusion products. Actually, fusion products were frequently found to be some morphological variants of the fusion parents (Yoo *et al.*, 1984; Yoo *et al.*, 1987; Toyomasu and Mori, 1987a; Kirimura *et al.*, 1989). The purpose of the experiment in this chapter was to provide the morphological and cytological information of both fusion parents and fusion products such that the fusion products were characterized with respect to their corresponding mycelial, fruit bodies and basidial morphologies as well as the number of nuclei in their mycelial cells.

5.2. Materials and methods

5.2.1. Strains

For characterization of the fusion products in the present research, the morphological and cytological characters of both the fusion parents (*Pleurotus florida* Pf67 and *Schizophyllum commune* Sc17) and the fusion products (PS1, PS2

and PS3) were studied. All the above fungal strains were maintained on PDA medium and incubated at 28 °C in darkness.

5.2.2. Studies on colonial and mycelial morphology

Mycelia of these fungal strains were inoculated onto PDA media in petri dishs. All cultures were incubated at 28 °C in darkness for 14 days. The morphology of the aerial hyphae as well as the coloration of the agar underneath the colony were inspected. Mycelial morphology of each fungal strain was studied through microscopic observations. Fungal hypha of the investigating species were picked along with the agar medium from the colonial margin and placed on a glass slide. The agar with fungal hyphae was covered and pressed with a coverslip. Microscopic examinations micrograph taking were carried out with the 400 × phase contrast light microscope [Zeiss].

5.2.3. Fluorescent staining of mycelial nuclei with DAPI

The nuclear staining method described here was a modified procedure of the one described by Meixner and Bresinsky (1988) and similar those described in section 4.2.. Both the fusion parents (Pf67 and Sc17) and the fusion products (PS1, PS2 and PS3) were studied in this section. Several tenths of cover glasses (2.4 cm × 3.6 cm) [Deckgläser] were autoclaved in distilled water. A sterilized cover glass was inserted at an inclined angle of about 10 degrees into the PDA medium inside a petri dish such that 1/3 of the cover glass was inserted into the agar. An agar block with vegetative mycelia of the investigating strain was inoculated onto the PDA medium by the side of the inserted cover glass. After five to seven days of incubation at 28°C in darkness, mycelia were grown onto the surface of the cover glass. The slide

was then removed from the agar medium and heat-dried in the 60°C oven for 30 minutes. Afterwards, mycelium fixation solution was then added onto the heat-fixed mycelia and chemically fixed for 15 minutes. The mycelia sample was then washed in McIlvaine's citrate / phosphate buffer pH 7.0 for another 15 minute. DAPI dye solution was then added to the mycelia sample for 15 minutes at 37°C. Then the mycelia sample was being washed again by McIlvaine's citrate / phosphate buffer pH 7.0 for 7 minutes. Then the excess solution on the mycelia sample was removed by mopping with tissue paper. The cover glass was placed onto the glass slide with the mycelia in between the cover glass and the glass slide. The specimen was then mount in glycerol.

For fluorescence microscopic observations and micrographs taking, the specimen was observed using a Nikon Biophot [Nikon] epifluorescent microscope equipped with 100-W mercury vapor lamp. Observations were made by using filter combination UV-1A, giving a peak of excitation light between 330 and 380. ILFORD HP5 negative films were used for micrographs taking.

5.2.4. Study on fruit body and basidial morphology

5.2.4.1.Fruiting on agar plate

Fruit bodies of three strains showed in table 5.1. were studied in this section. Corresponding compatible monokaryons were inoculated onto PDA medium three to five millimeters apart. Dikaryosis was judged by the occurrence of true clamp connections. Dikaryotic mycelia were transferred onto another PDA medium and maintained at 28 °C as the stock dikaryotic strain for the corresponding fungus.

Table 5.1. The dikaryotic strains for fruit body formation and their corresponding sources.

Fungal strains	Formation of the corresponding dikaryon
Schizophyllum commune (Sc17)	Sc17 × Sc 4
Pleurotus florida (Pf67)	Pf67 × Pf 4
Fusion product PS1	Protoplast fusion of Sc17 and Pf67

For *Pleurotus florida*, dikaryotic culture was incubated at 28 °C for two weeks in darkness. The culture was then further incubated in light dark cycle with 8 hours light and 16 hour darkness for four weeks to form fruit body at 25 °C. Aeration of the culture was increased by removing the parafilm wrap. For *Schizophyllum commune*, the dikaryotic culture was incubated for 7 days. Fruit body formation was initiated by increasing aeration and light stimulation as in *Pleurotus florida*. However, the incubation time for mature fruit body formation was 4 days. The crosses between *Pleurotus florida* Pf67 and *Pleurotus florida* Pf4 as well as *Schizophyllum commune* Sc17 and *Schizophyllum commune* Sc4 fruited normally on PDA medium in petri dish at 25 °C. For dikaryotic fusion product PS1, the culture was inoculated onto PDA plate and incubated at 28 °C for 7 days until the mycelia of the colony reached the culture plate margin. Stimulation for fruiting then started. Aeration of culture was increased by removing the parafilm wrap. The plate was placed inside an incubation chamber with light dark cycle of 8 hours light and 16 hours dark for 6 days (Schwalb, 1978). The environmental temperature for

fruiting was the same as for both *Schizophyllum commune* and *Pleurotus florida* (25 ^{o}C).

5.2.4.2.Scanning electron microscopic examination

For comparative studies, the morphologies of basidiospores and basidia of the fruit body of PS1, *Schizophyllum commune* and *Pleurotus florida* were observed. Scanning electron microscopy was carried out for the three strains. The method used was a similar to the one described by Chiu and Chang (1987). Fresh fruit body of the investigating strain was collected. Pieces of gill were dissected into 2 mm × 5 mm squares. The gill fragments were fixed with glutaraldehyde fixation solution for 1.5 hours in vacuum. Afterwards, the samples were placed into a fresh 6% glutaraldehyde fixation solution and fixed for another 2 hour in vacuum. The glutaraldehyde fixation solution on specimen was then rinsed away with 0.1 M Sorenzen's phosphate buffer pH 7.2. Post-fixation was then carried out by submerging the sample into the 2 % osmium tetroxide in 0.1 M Sorenzen's phosphate buffer pH 7.2. in a fume cupboard for 1 hour. The same was then rinsed with 0.1 M Sorenzen's phosphate buffer pH 7.2 again. After fixation, specimens were dehydrated via up-graded alcohol series (30%, 50%, 70%, 95%, 100% and 100% ethanol). For each alcohol solution, the specimens were submerged for 30 minutes in vacuum. After completed the dehydration steps, critical point drying (Ladd Research Industries, Inc.) was carried out at 1,300 psi and 40 ^{o}C using liquid nitrogen as the sublimation medium. The specimens were sputter-coated with gold and observed by scanning electron microscope (JOEL model JSM-35, JOEL, TOKYO, JAPAN).

5.3. Results

5.3.1. Variation of colonial morphology

The colonial morphology of fusion parents (*Pleurotus florida* Pf67, *Schizophyllum commune* Sc17) and fusion regenerants (PS1, PS2 and PS3) with the same culture age (14 days) were shown in figure 5.1.. For Pf67 and PS3, both showed to have similar colonial morphologies. They showed to have compact aerial mycelia and able to produce metabolic substance that stained the agar medium yellowish brown. Although, such brown coloration was found to be most intensive in agar at the base of the corresponding inocula, the stained region was not restricted to the area underneath their colonies but the whole agar media of the corresponding cultures. Both of their colonies showed to have a quite defined colonial margin. However, sector-like morphology was observed in the PS3 colonies. For Sc17, the colony showed to have limited aerial hyphae and relatively richer in submerged hyphae. The agar underneath the colony was stained pale purplish pink. Unlike the situation in Pf67 or PS3, the coloration was restricted to be underneath the area covered by the colony. The colony showed to have a fuzzy margin and the hyphal arrangement was quite loose. For PS2, the colony showed no agar stained character. The colony was rich in aerial hyphae like Pf67 but the hyphal arrangement was rather loose and comparable to the submerged hyphae of Sc17. Similar to Sc17, colony of PS2 showed to have a fuzzy margin. For PS3, the colony showed no agar stained character of the two fusion parents. However, it showed to form hyphal mat on the agar surface and had a fuzzy colony margin. PS1 showed to have compact aerial hyphae and no pigmentation was observed in the agar of the culture. Colonial margin was fuzzy as that of Sc17 colony. Unlike Sc17, PS1 showed to have limited among of submerged hyphae.

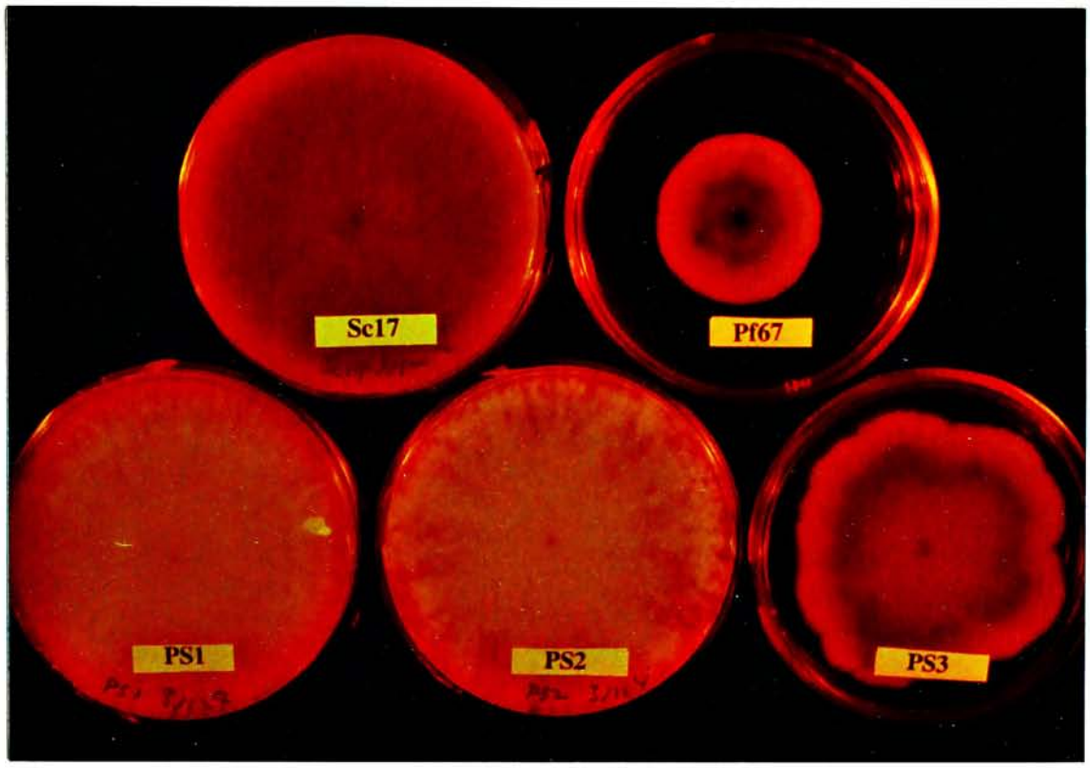


Figure 5.1. The colonial morphology of the fusion parents and fusion products. Pf67 - *Pleurotus florida*; Sc17 - *Schizophyllum commune*; PS1, PS2, PS3 - three fusion products. The photograph showed the bottom view of the cultures.

5.3.2. Morphologies and the number of nuclei in the mycelial cells of fusion parents and fusion products

The mycelial morphologies of the five strains were shown in figure 5.2. to figure 5.6.. The mycelium of Sc17 (figure 5.3.A) was frequently curved along the whole length of mycelium. However, mycelia of Pf67 showed to be relatively straighter (figure 5.2.A) than that of Sc17. Mycelia of PS1 have clamp connections and curved frequently (figure 5.4.A) as in Sc17. For the other two fusion products, PS2 (figure 5.5.A) and PS3 (figure 5.6.A), both of them showed to have straight mycelium as those of Pf67.

DAPI staining showed that only PS1 (figure 5.4.B) was dikaryotic. All of the two fusion products and the two fusion parents were monokaryons. The two nuclei of the cell in PS1 were in close proximity and septum was observed in the clamp connection. Nuclei of Pf67 showed to be oval shape (figure 5.2.B). However, nuclei of Sc17 were elongated shape (figure 5.3.B). The shape of nuclei of PS2 was similar to those of Sc17 (figure 5.5.B) which was elongated shape. Nuclei of PS3 showed to be mostly oval shape which was similar to those of Pf67, however, some of them were elongated (figure 5.6.B).

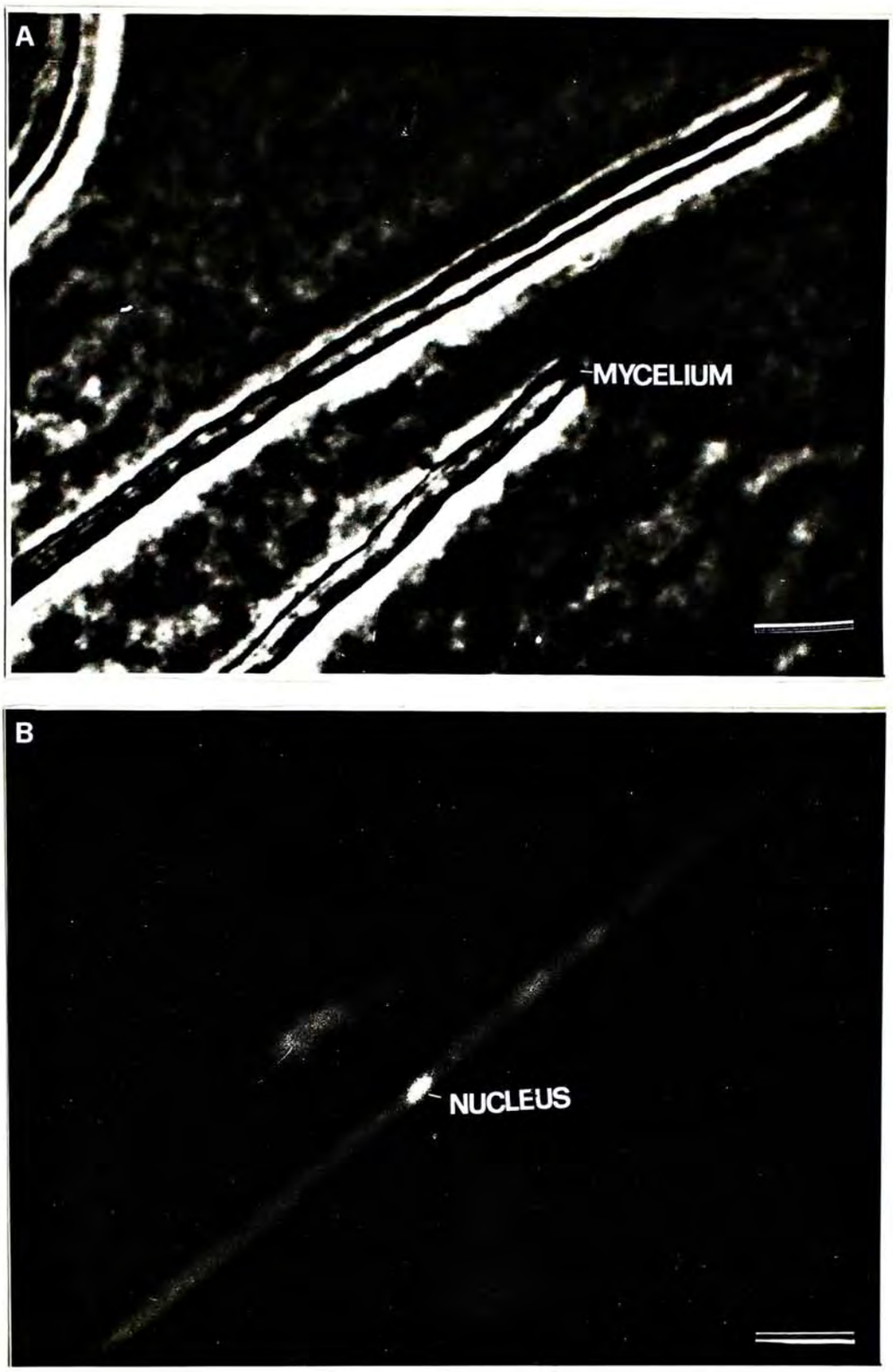


Figure 5.2. Micrographs showing the number of nuclei and the morphologies of the mycelial cells of fusion parent *Pleurotus florida* Pf67. (A) Light micrograph of the mycelia. (B) Fluorescence micrograph showing the number of nuclei in mycelial cells. (scale bar = 5 μm)

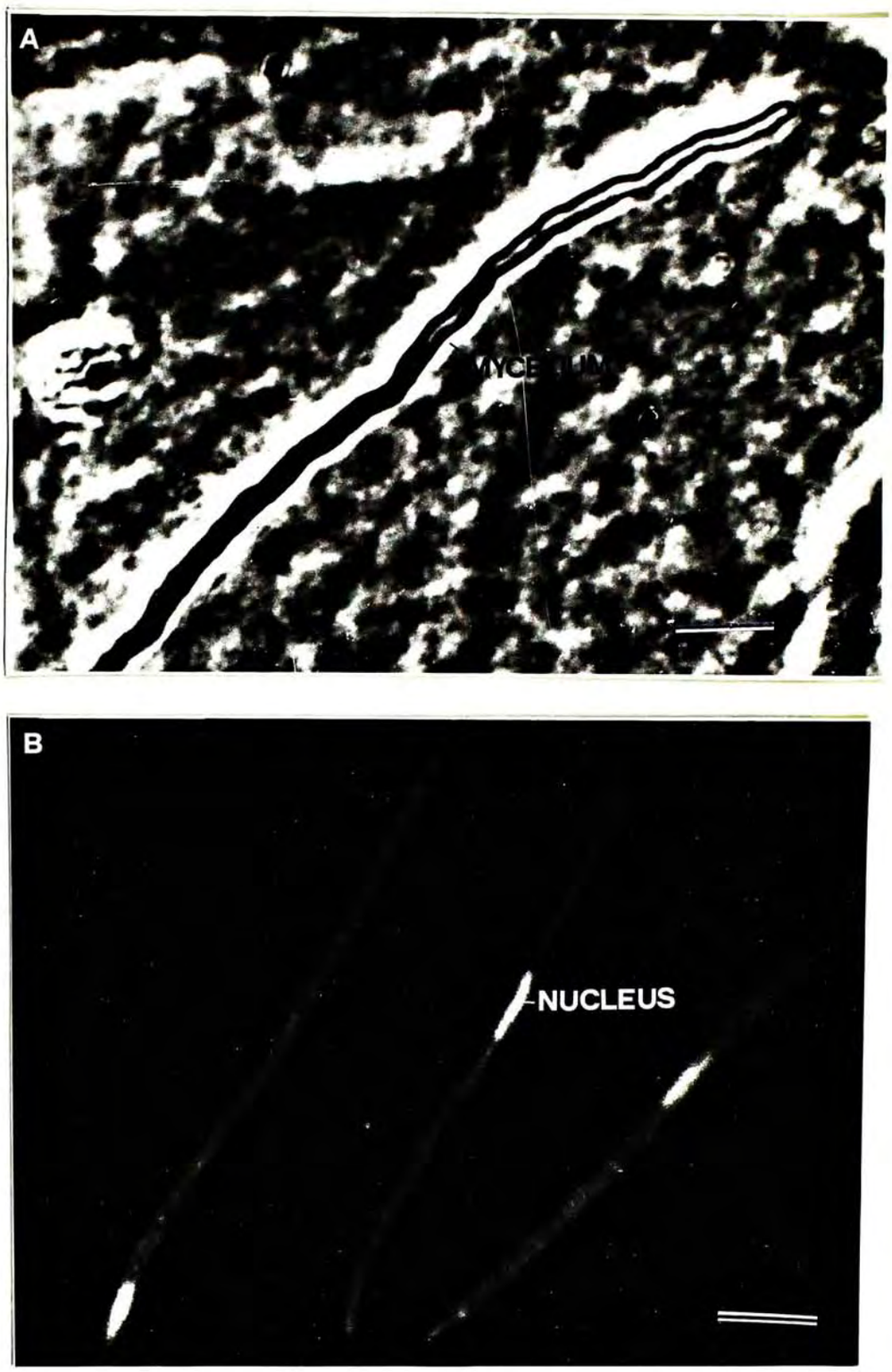


Figure 5.3. Micrographs showing the number of nuclei and the morphologies of the mycelial cells of fusion parent *Schizophyllum commune* Sc17. (A) Light micrograph of the mycelia. (B) Fluorescence micrograph showing the number of nuclei in mycelial cells. (scale bar = 5 μm)

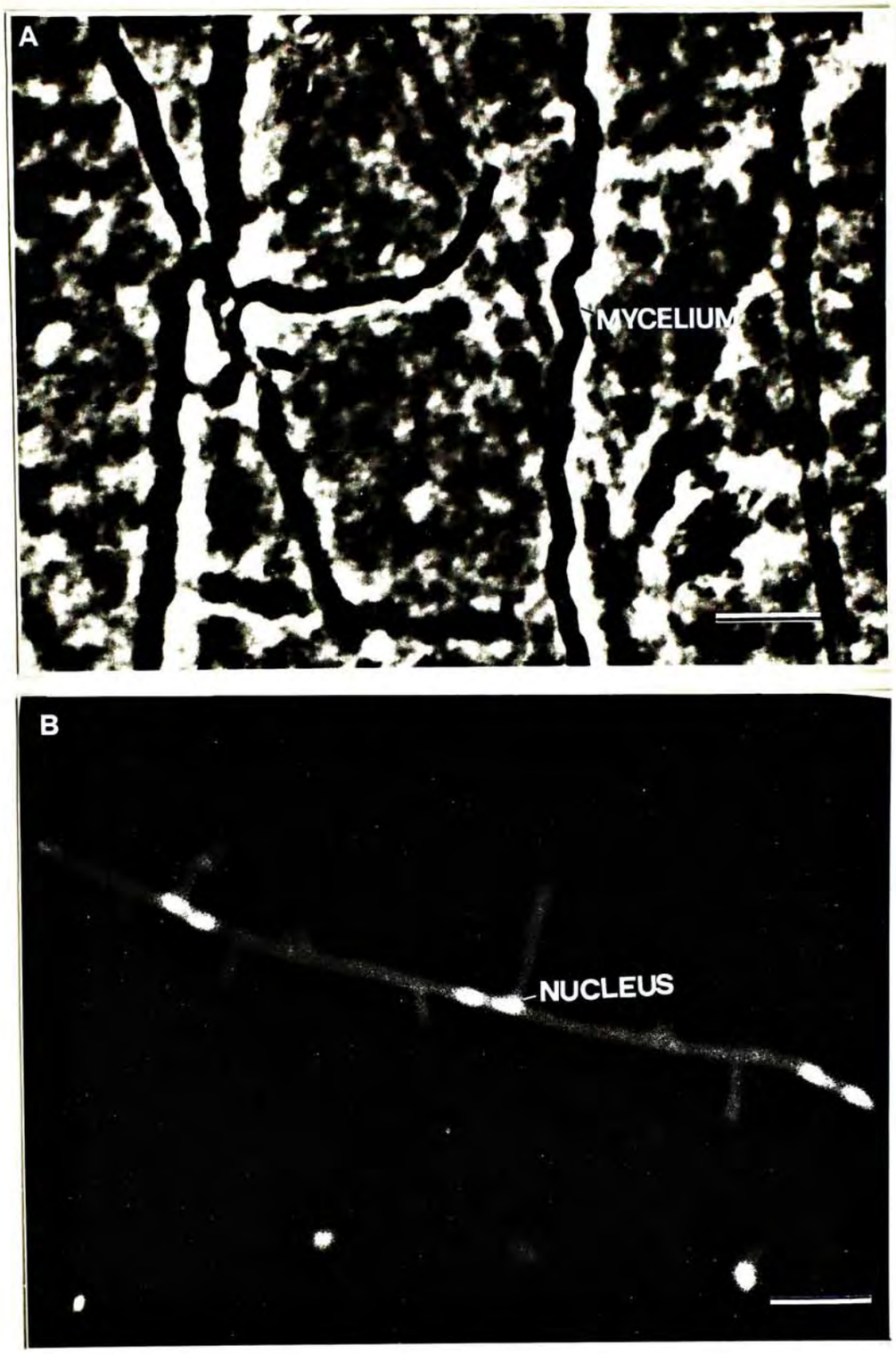


Figure 5.4. Micrographs showing the number of nuclei and the morphologies of the mycelial cells of fusion product PS1. (A) Light micrograph of the mycelia. (B) Fluorescence micrograph showing the number of nuclei in mycelial cells. (scale bar = 5 μm)

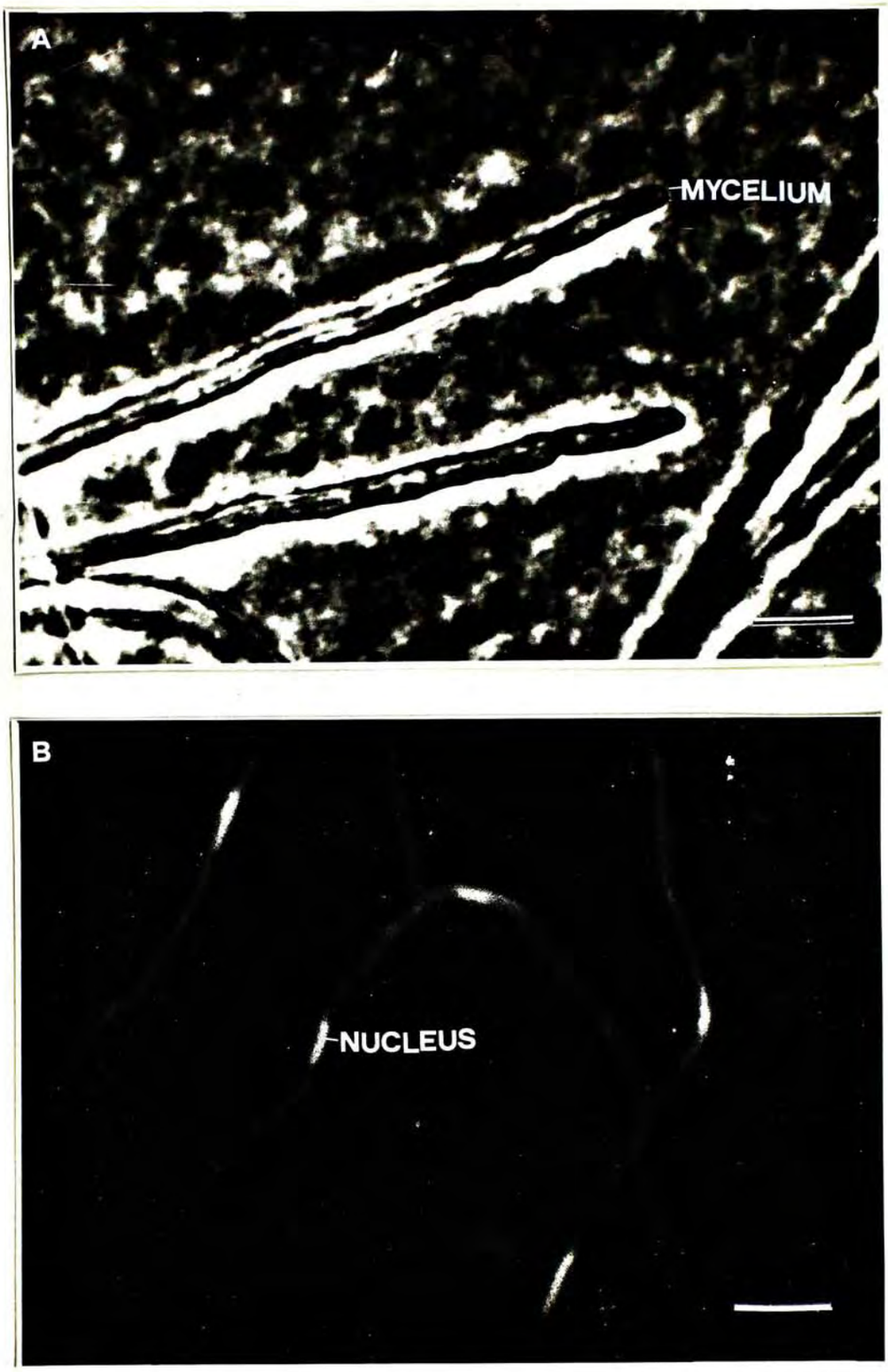


Figure 5.5. Micrographs showing the number of nuclei and the morphologies of the mycelial cells of fusion product PS2. (A) Light micrograph of the mycelia. (B) Fluorescence micrograph showing the number of nuclei in mycelial cells. (scale bar = 5 μm)

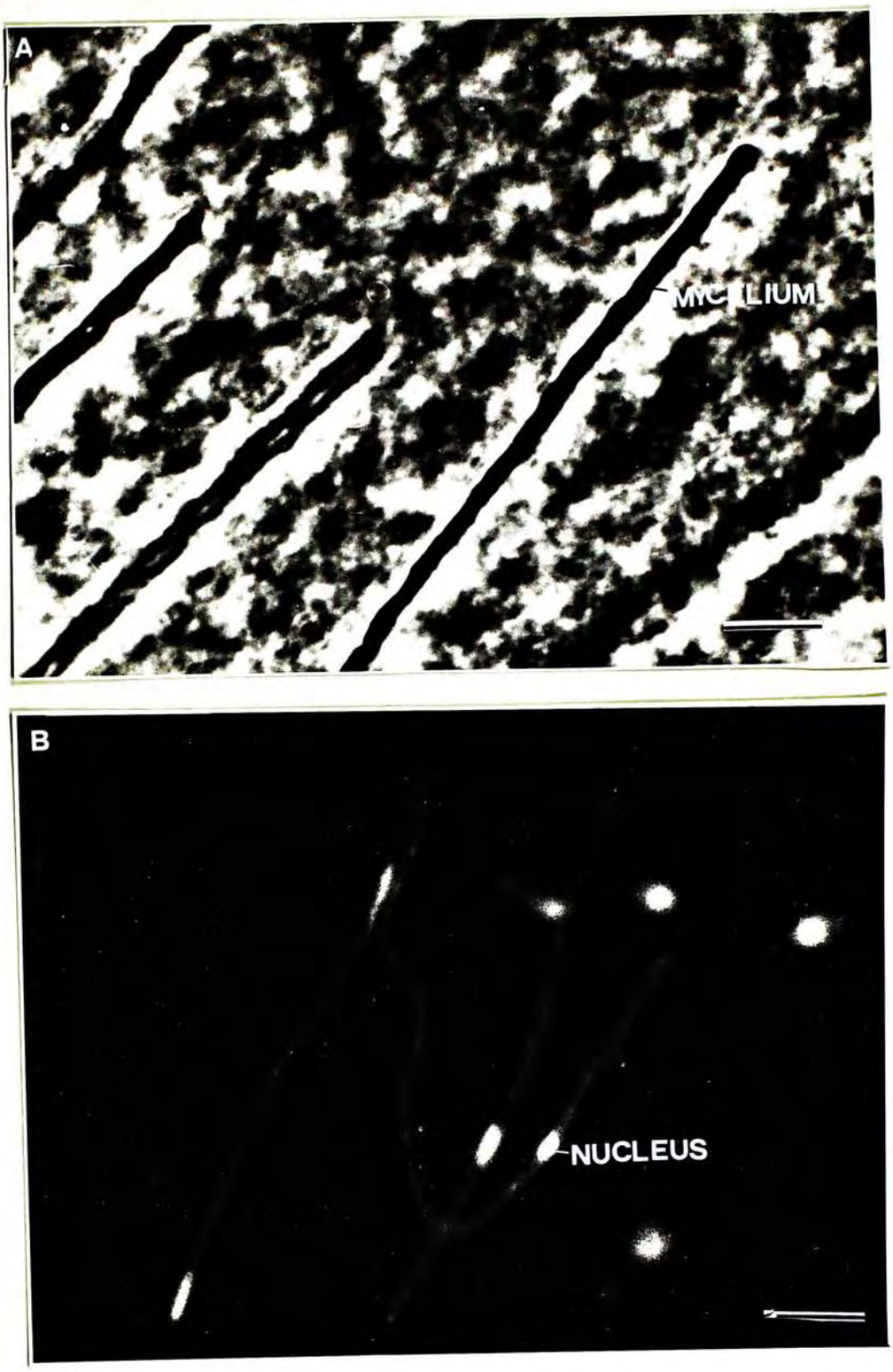


Figure 5.6. Micrographs showing the number of nuclei and the morphologies of the mycelial cells of fusion product PS3. (A) Light micrograph of the mycelia. (B) Fluorescence micrograph showing the number of nuclei in mycelial cells. (scale bar = 5 μm)

5.3.3. Fruit body morphology

Morphologies of fruit bodies of the *Pleurotus florida, Schizophyllum commune* and PS1 were shown in figure 5.7.. Fruit bodies of the three fungal strains were different on the aspects of the length of stalk, the orientation of gill. For *Pleurotus florida*, the stipe of fruit body was long and the gill was developed on the bottom surface of the pileus (figure 5.7.A). However, there was generally no observable stipe growth or very short stipe in fruit body of *Schizophyllum commune* (figure 5.7.B). Moreover, gill of *Schizophyllum commune* fruit body was developed on the upper surface of the pileus. For fruit body of dikaryotic fusion product PS1, the orientation of gill was similar to that of *Schizophyllum commune* but the stipe was thick and long as that of Pf67 (figure 5.7.C).

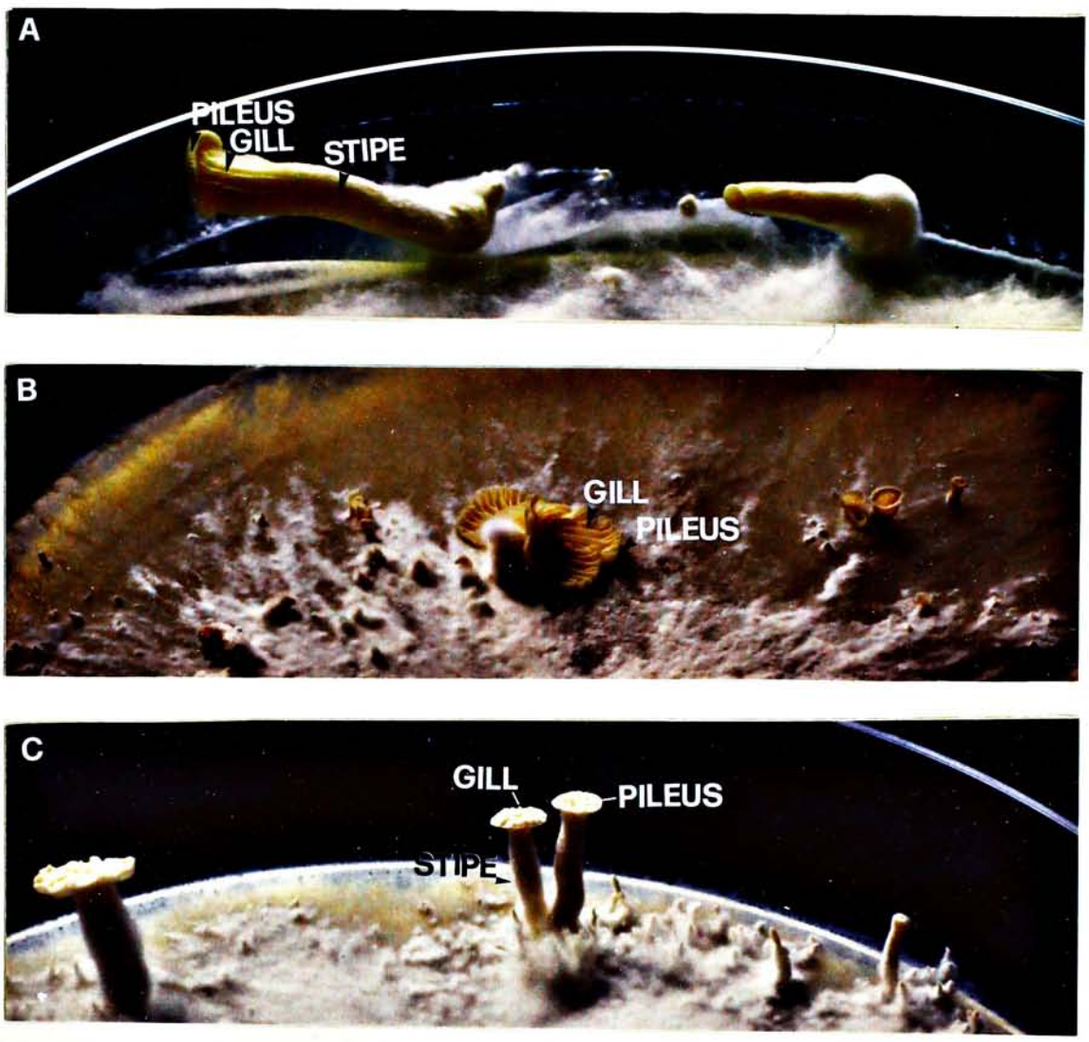


Figure 5.7. Fruit body morphologies. (A) *Pleurotus florida*, (B) *Schizophyllum commune* and (C) dikaryotic fusion product PS1.

5.3.4. Basidial morphology

The basidia and basidiospores were shown in the electron micrographs of figure 5.8. to figure 5.9.. Morphology of the basidia and basidiospores of *Pleurotus florida* (figure 5.8.A and B) and *Schizophyllum commune* (figure 5.8.C and D) showed to be quite similar. The basidia of both strains showed to be able to form four basidiospores which were kidney shape. However, the sterigmata of *Schizophyllum commune* (figure 5.8.D) were relatively longer than those of *Pleurotus florida* (figure 5.8.B). The lengths of sterigmata of *Schizophyllum commune* and *Pleurotus florida* were approximately equal to 3 μm and 1.5 μm respectively. Similar to the two fusion parents, PS1 was able to develop tetrasporal basidium and the basidiospores were kidney shape (figure 5.9.A). The sterigmata of PS1 were rather long and had similar dimension with those of *Schizophyllum commune* (figure 5.9.B).

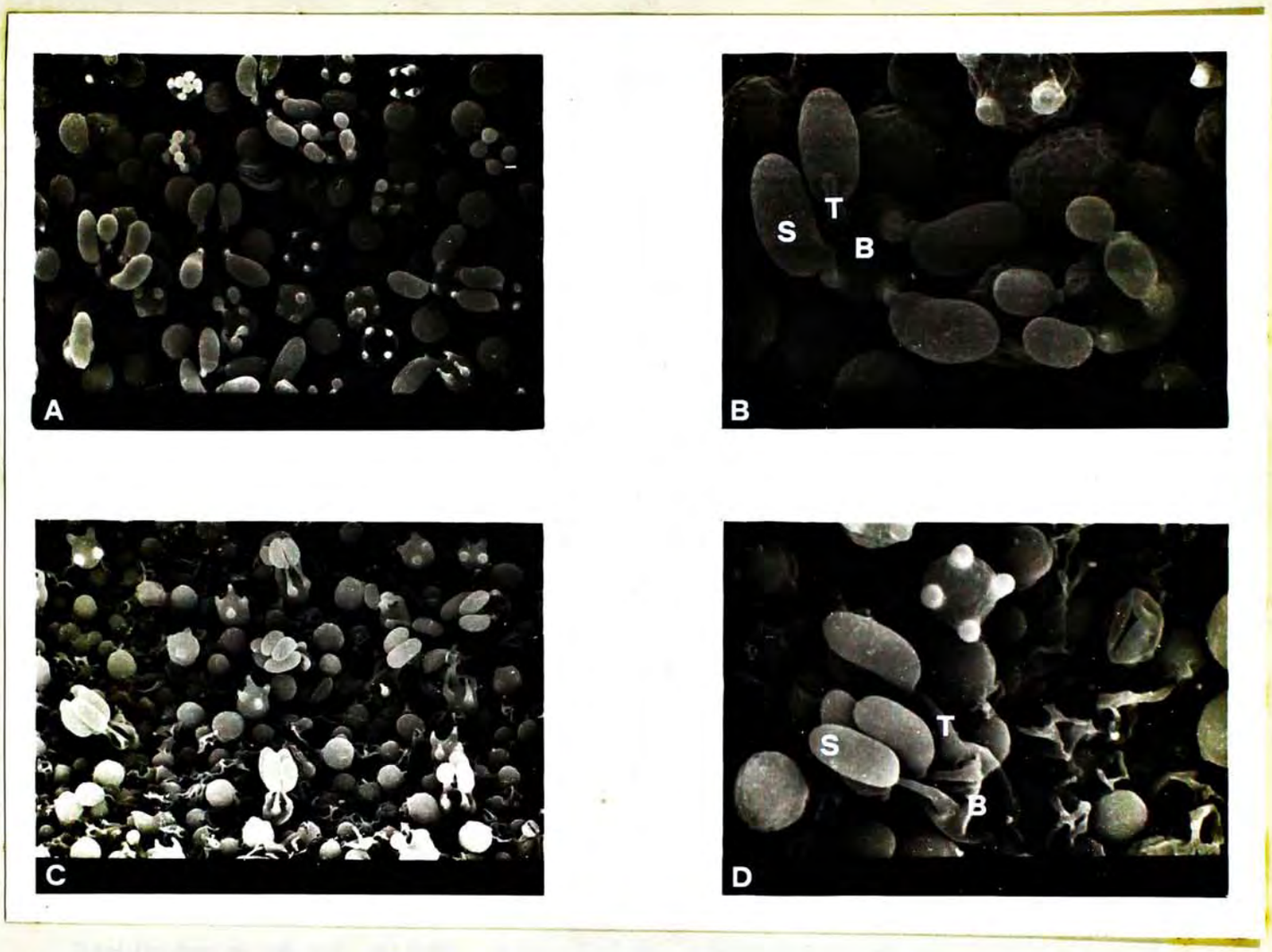


Figure 5.8. Scanning electron micrographs showed the morphologies of basidia and basidiospores. (A) and (B) - *Pleurotus florida,* 2400 × and 7200 × respectively. (C) and (D) - *Schizophyllum commune,* 3000 × and 7800 × respectively.. Figure (A) and (C) showed that all matured basidia of the two strains produced four basidiospores. Figure (B) and (D) showed that the sterigmata of *Pleurotus florida* was relatively shorter than those of *Schizophyllum commune* and estimated to be 1.5 μm and 3 μm respectively. (S - basidiospore, T - sterigma, B - basidium)

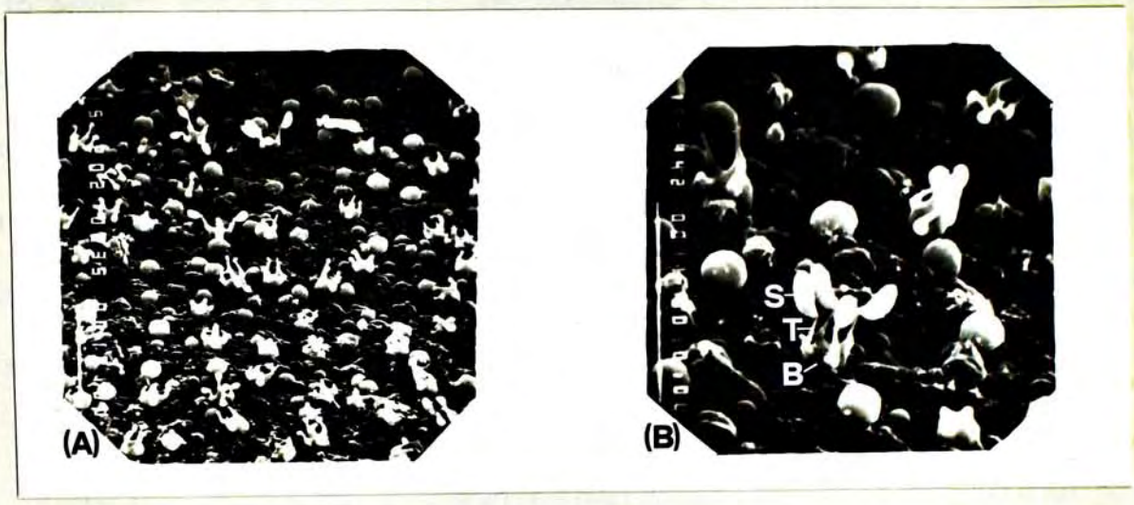


Figure 5.9. Scanning electron micrographs showed the morphologies of basidia and basidiospores of dikaryotic fusion product PS1. (A) Numerous basidia and basidiospores on gill surface. Each mature basidium was able to produced four basidiospores (2020 ×). (B) Enlarge view of basidium and basidiospores showed that the sterigmata of PS1 were quite long. (S - basidiospore, T - sterigma, B - basidium)

5.4. Discussions

The presence of clamp connections and the binucleated mycelial cells confirmed the dikaryotic nature of the fusion product PS1 (figure 5.4.B). The formation of true clamp connections on PS1 mycelia implied the A≠B≠ relationship of the incompatibility factors in the two nuclei in each PS1 mycelial cell. In fruiting condition similar to those for the two parents, dikaryotic fusion product PS1 was able to develop mature fruit body. Morphological differences were recognized in appearance among the fruit bodies of the dikaryotic fusion product PS1 and the two fusion parents. Dikaryotic fusion product PS1 had *Schizophyllum commune*-like pileus and the location of the gill development was also similar to that of *Schizophyllum commune*. However, the development of long stipe of PS1 fruit body was similar to that of *Pleurotus florida*.

Long stipe fruit body of *Schizophyllum commune* (*MED* mutant) had been used for studying the regulation of fruiting (Schwalb, 1978). As the fruit body morphology of *MED* mutant was similar to that of PS1, the genetical changes in PS1 lead to the formation of long stalk in fruit body might be similar to that of *MED* mutant. The expression of the *med* mutation phenotype was explained as a result of extensive differentiative growth in the differentiated state of stipe formation. Actually, Schwalb suggest that the phenomenon was a result of interruption of the developmental sequence by the increase in sensitivity to environmental stimuli. On the other hand, Schwalb also suggested that there were no interlocking controls between different differentiation stages. Therefore, the expression of the signal did not require the normally intervening events to take place. Although the exact genetical change(s) which caused for the formation of long stipe in PS1 fruit body was still unknown, Schwalb's studies showed some implications on the above aspect.

That is, the genetical changes in fusion product after PEG fusion treatment might be correlated with the changes in genetical material responsible for receiving the specific environmental signal in stipe formation. Therefore, the corresponding preceptivity might be changed. Consequently, differentiative growth of stipe might be resulted. Comparison of basidia and basidiospores of PS1 and *Schizophyllum commune* showed no observable morphological difference. Therefore, change in genetic materials of PS1, if present, might be occurred in DNA sequences which was not related to the morphogenesis of basidiospores.

As long as obtaining a fusion product PS1, which was able to form fruit body with mutant phenotype through protoplast fusion process, it was valuable for us to direct our research towards understanding the mechanisms of the genetic materials' interaction during or after intergeneric protoplast fusion process.

Considering the shape of the nucleus of PS2 (figure 5.5.B), the elongated shape was similar to that of Sc17. However, the morphology of this fusion product was intermediate between the two fusion parents (figure 5.1.). It was because the fuzzy margin and the loose mycelial arrangement of colony were similar to that of Sc17 but the colony was rich in aerial hyphae which was similar to that of Pf67. In addition, the mycelial morphology of PS2 was similar to that of Pf67 because mycelia of PS2 were quite straight.

The sector-like morphology of the PS3 colony (figure 5.6.A) might reflect a genetic variation among the cells in the colony. Therefore, a certain extent of genetic instability might be present in this fusion product PS3. On the aspect of the shape of nucleus, the infrequently occurrence of Sc17-like elongated nuclei in PS3 might be due to the transient stage of nuclear division because variation of nucleus shape in

different nuclear stages has been reported (Meixner and Bresinsky, 1988). However, the exact reason for the above phenomenon was unclear so far, we still could not eliminate the possibility that the phenomenon was related to the genetic instability of the strain which was implied from the colonial morphology.

From all of the above results we could see a morphological variation in the fusion products with respect to the two fusion parents. Although the exact mechanism causing such variations was still unknown, the morphological differences and cytological variations might indication a change in genetic materials of the parental genome through the protoplast fusion process. More evidence for proving the possibility of genetical changes in the fusion products relative to the two fusion parents would be given by using molecular technique as shown in chapter 7.

Chapter 6

Physiological Studies of Fusion Parents as well as Fusion Products by Investigating the Growth Responses to Drugs

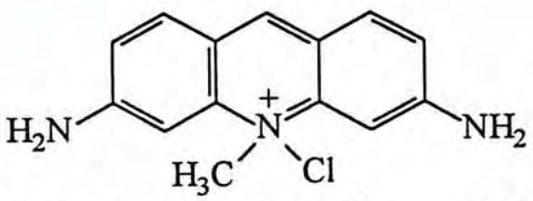
6.1. Introduction

As stated in table 3.1. in chapter 3, both fusion parents (Pf67 and Sc17) have drug resistance markers. That is, the segregation pattern of these genetic markers, which is expressed in the fusion products, can provide some information about the exchange of genetic materials through the process of protoplast fusion. Actually, physiological changes such as improvement of certain metabolic products in the fusion products have been reported. The improvement of cephalosporin C titer in recombinants of two high titer strains of *C. acremonium* that had been crossed using the fusion technique (Hamlyn and Ball, 1979) was an example. However, not all breeding crosses have been so encouraging. Kirimura *et al.* in 1987 were unsuccessful in improving the citric acid production in *A. niger* through protoplast fusion. Therefore, physiological changes, which were related to the susceptibility to the two drugs (acriflavin and guaiacol), present in the fusion products of the present study was revealed by studying the growth pattern of the fungal strains on the complete agar medium with different concentrations of either drug.

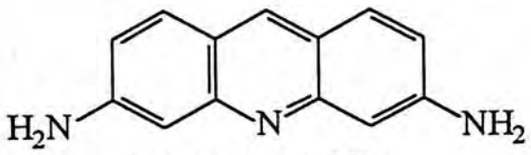
Acriflavin and guaiacol were used as the two fungitoxic drugs in this part of study. Antifungal action of acriflavin was found to be parallel to the degree of ionization (figure 6.1.) (Lukens, 1969). Moreover, the hydrocarbon substituents in the structure of acriflavin able to increase the permeation of drug into the cell of the organism. The presence of polar groups increases the chemical activity (toxicity) of the drug to the organism since this properties aid adsorption of the dye on to the

receptor surface. The receptors have been identified as nucleic acids by Gurr et al in 1974. However, the toxic effect of the drug was due to the heterocyclic nitrogen nucleus. Therefore, the toxicity of acriflavin should mainly due to the polar component, 3,6-diamino-10-methylacridinium chloride. The fungicidal action of the drug was based on its ability to ionize cell membrane and led to the leakage of cellular constituents. Hence, a wide spectrum of physiological functions, such as the electron transport system, activators of enzyme, cell membrane as well as organelle structures, were being affected. Such kind of interruptions on the physiological process would eventually lead to cell death (figure 6.2.).

Acriflavin



3,6-diamino-10-methylacridinium chloride



3,6-diaminoacridine

Guaiacol

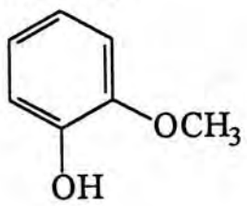


Figure 6.1. The chemical structures of acriflavin and guaiacol.

On the aspect of fungitoxicity of guaiacol, it was showed to have a fungistatic effect. Although the primary site of action was still unknown, the mechanism of the fungistatic effect of guaiacol was generally considered to involved a non-specific binding of the chemical onto cell wall as well as membrane (Lyr, 1977). After penetration and dissociation of guaiacol, it causes an uncoupling effect on phosphate metabolism and finally blocked the electron transport system (figure 6.3.).

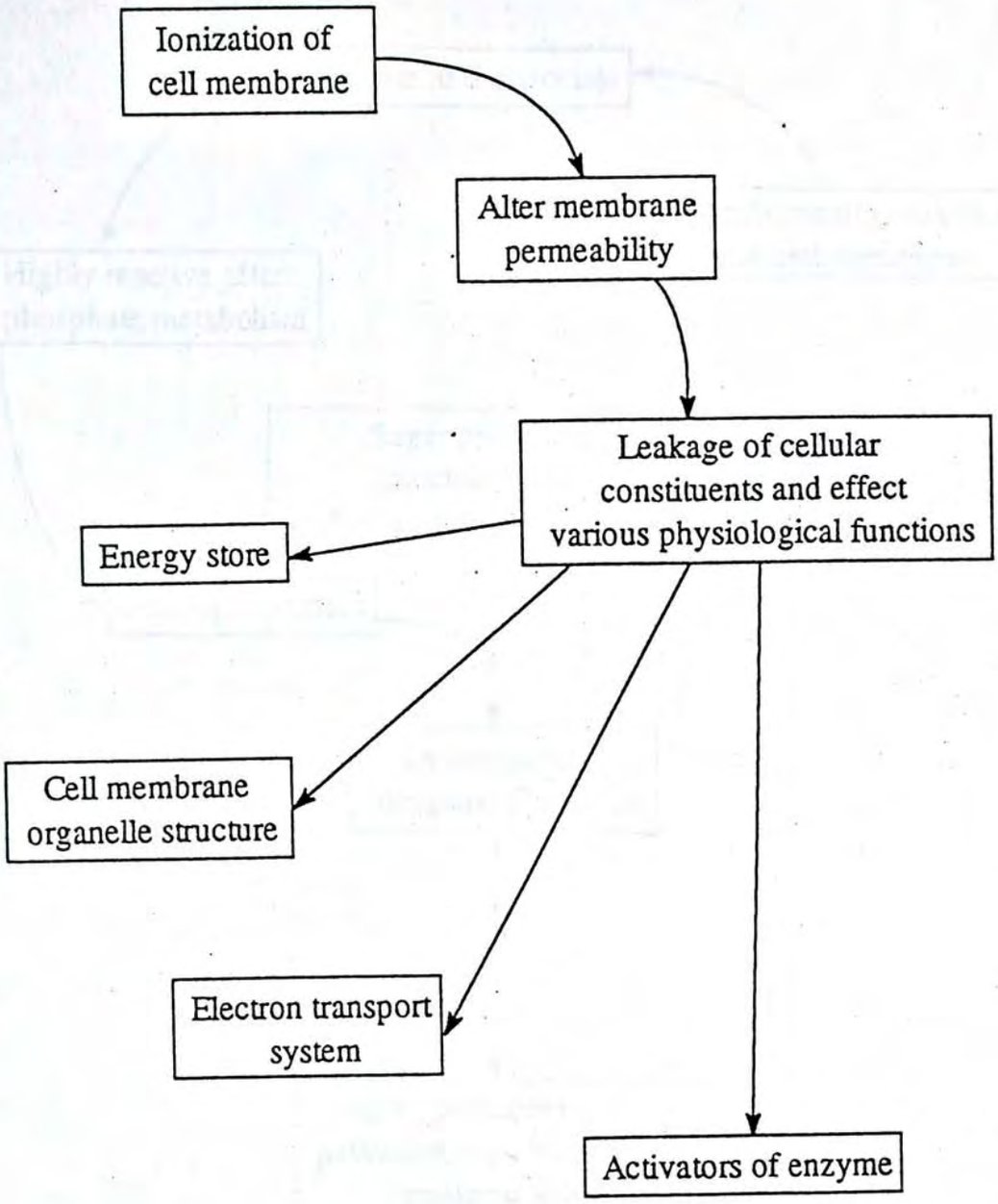


Figure 6.2. Simplified mechanism of acriflavin as a fungicidal drug.

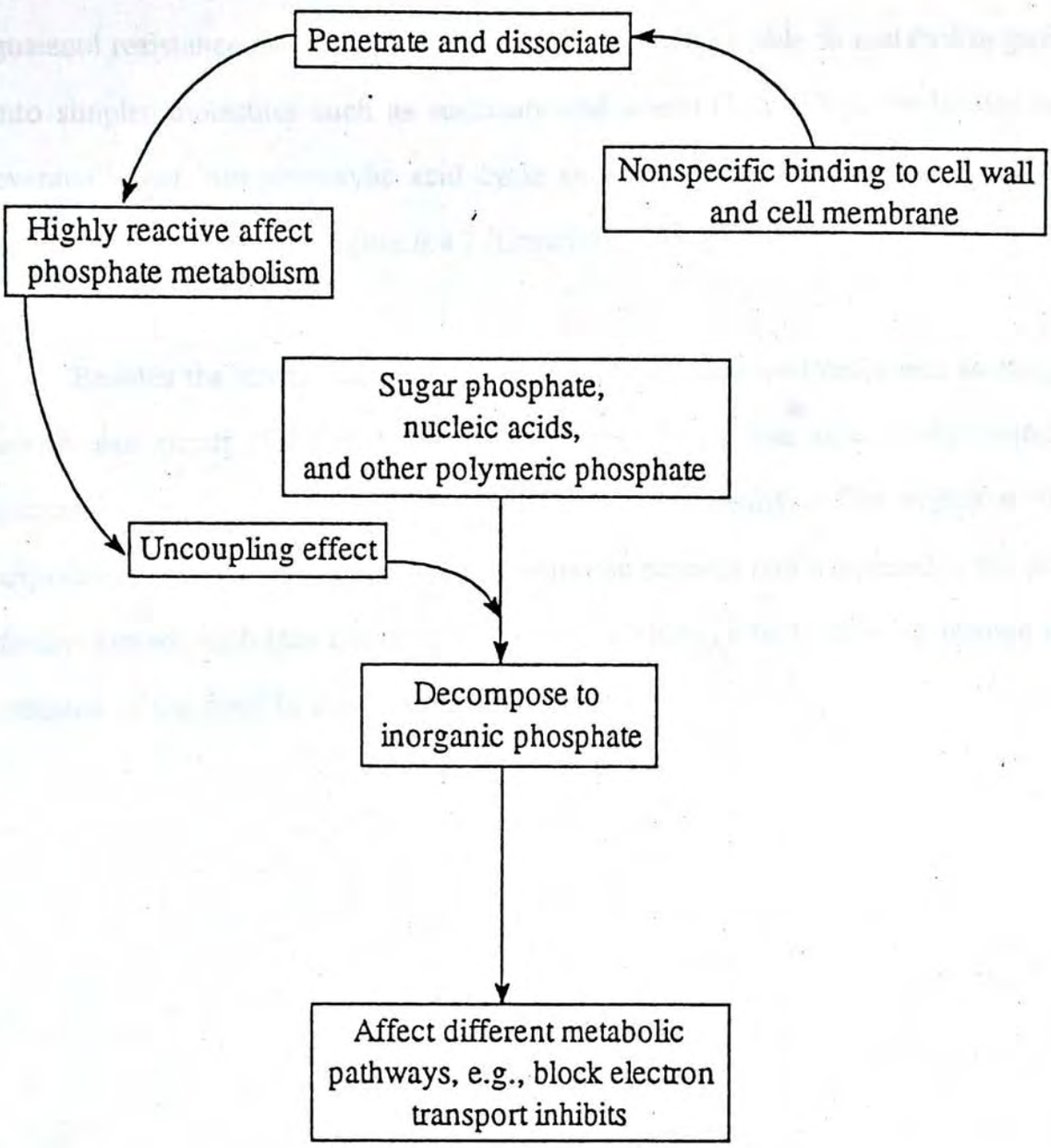


Figure 6.3. Fungistatic effects of guaiacol. The primary site of action is still unknown.

As stated in table 3.1., Sc17 was able to grow on the medium with 100 μg/ml acriflavin or 1 μg/ml guaiacol. Therefore, Sc17 might have the acriflavin and guaiacol resistance mechanism. Actually, Sc17 might be able to metabolize guaiacol into simpler molecules such as succinate and acetyl-CoA. These molecules would eventually get into glycoxylic acid cycle and was being transformed into different carbohydrate molecules (figure 6.4.) (Crawford, 1981).

Besides the innate fungicide resistance ability, acquired resistance to fungicide could also occur (Griffin, 1981). Protoplast fusion was one of the mutational processes for the fungi to acquire such resistance ability. The objective of this experiment was to investigate whether mutation process had happened in the present fusion system such that the resulting genetical changes had caused a change in the respond of the fungi to the two fungitoxic drugs.

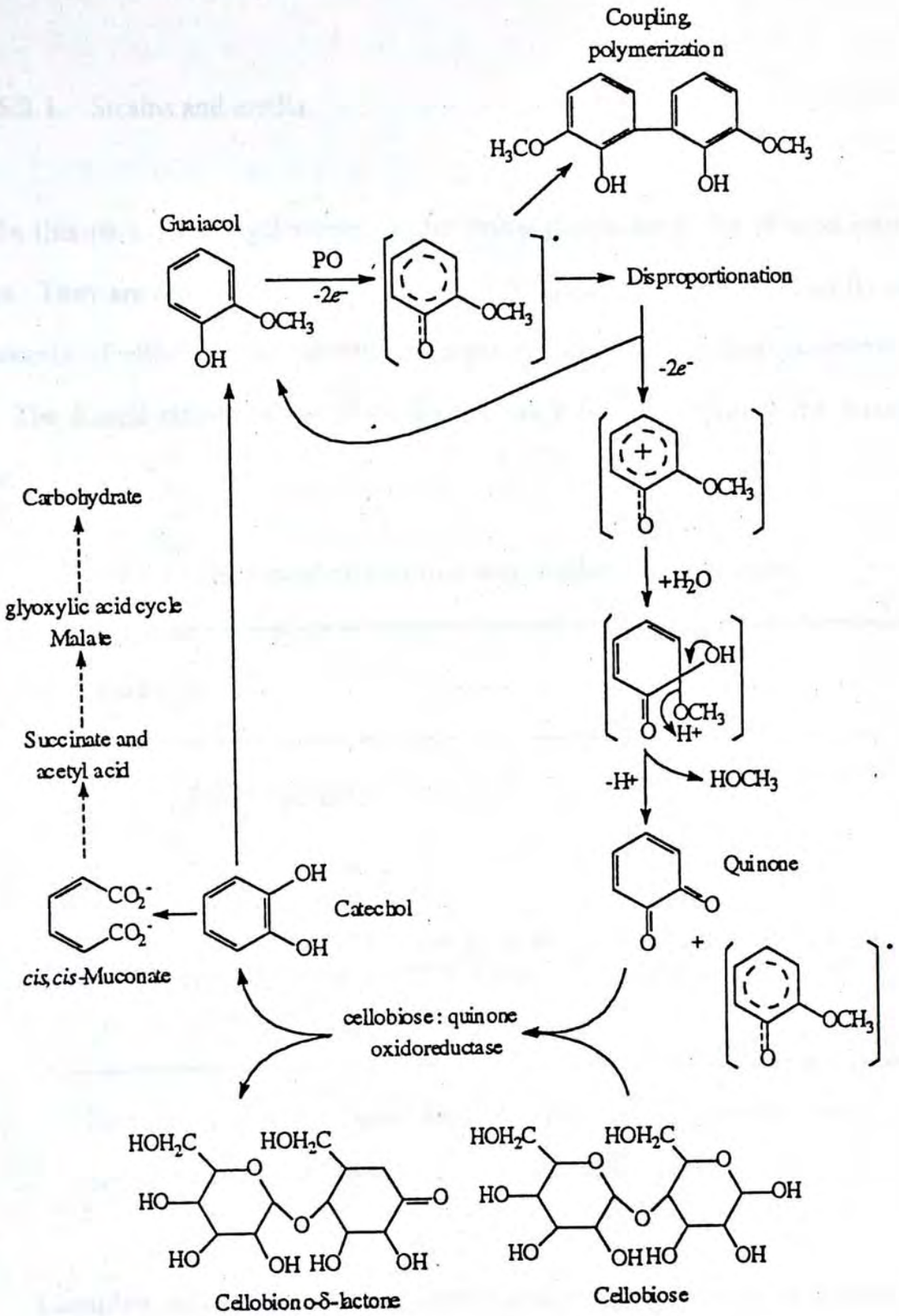


Figure 6.4. Some possible metabolic pathways of guaiacol in mycelium.

6.2. Materials and methods

6.2.1. Strains and media

In this part, the fungal strains under investigation could be divided into three groups. They are the fusion parents (group I), the fusion products (group II) and the regenerants of either fusion parents' protoplasts after PEG fusion treatment (table 6.1). The fungal strains of the three groups were maintained on PDA medium as stocks.

Table 6.1. The fungal strains that was studied in this chapter.

Groups		Fungal strains
I:	Fusion parents	Pf67, Sc17
II:	Fusion products	PS1, PS2, PS3
III*:	Regenerants of fusion parents' protoplasts after PEG fusion treatment.	From Pf67 : P1, P2, P3 From Sc17 : S1, S2, S3

*Remarks : Three regenerants of each fusion parents were obtained from the experiment of section 4.2.3.2..

Complete media added with different amounts of acriflavin or guaiacol were used as the culture media. The nutritional content of complete medium was the same as that described in section 4.2.3.1. of chapter 4. For determining guaiacol resistance marker, 1.11 mg/ml guaiacol stock solution was used. The stock solution was sterilized by millipore membrane filter as mentioned in section 4.2.3.1.. Ten

microliters of the stock solution was added to 1 liter of molten CM to produce a guaiacol agar medium with final concentration of 0.01 μg/ml guaiacol. By similar method, 100 μl, 1 ml and 10 ml stock solution were added separately to three 1 liter molten CM agar portions to produce guaiacol media with final concentration of 0.1, 1, and 10 μg/ml guaiacol media respectively. On the other hand, CM without adding guaiacol was also prepared. Thus the tested guaiacol concentrations were 0, 0.01, 0.1, 1 and 10 μg/ml. For the preparation of acriflavin medium, 100 mg/ml acriflavin stock solution was prepared as described in section 4.2.3.1.. The stock was sterilized by millipore filter as the guaiacol medium. Complete media with acriflavin concentrations of 0, 0.001, 0.01, 0.1 and 1 mg/ml were prepared by adding 0, 0.01, 0.1, 1 and 10 ml acriflavin stock solution into five 1 liter molten complete medium agar portions respectively.

6.2.2. Growth responses of the strains to different concentrations of drugs

For each strain, ten CM plates each contain different concentration of drug, that is, 0, 0.01, 0.1, 1, and 10 μg/ml for guaiacol agar medium and 0, 0.001, 0.01, 0.1 and 1 mg/ml for acriflavin agar medium, was prepared as mentioned in section 6.2.1.. One mycelium disc, which was 2 mm in diameter and approximately 1 mm thick, of the fungal strain was inoculated onto each the drug medium plate. Three replicas were prepared for the test of each drug concentration. As there were two types of drug media and each of them had five different concentrations, therefore, totally 30 plates were inoculated for each strain. The inoculated drug media plates were then incubated at 28^{o}C in darkness for 7 days.

The mean colony diameter of the culture in millimeter on each plate was recorded every 24 hours' interval for 7 days. For recording the diameter of a colony,

a mean diameter of each colony was calculated from four measurments. For each measuring, the plate was turned 45 degrees clockwise with the inoculum as center and another measuring was then made. The growth pattern was then recorded by plotting the colony diameter against the accumulated incubation time. The corresponding mean growth rate of each strain on different media from day 3 to day 7 were calculated by the following formula.

$$\text{Mean growth rate} = \frac{*\frac{\text{Day 4 - Day 3}}{1} + \frac{\text{Day 5 - Day 4}}{1} + \frac{\text{Day 6 - Day 5}}{1} + \frac{\text{Day 7 - Day 6}}{1}}{\text{4 days (day)}}$$

*Remark : Day = colony diameter on the corresponding day.

Statistical analysis for comparison of different data points was determined by independent t-test (Sigma plot version 5.0) at P = 0.05 level to notify the present of significant differences.

6.3. Results

6.3.1. Comparison of growth pattern as well as growth rate between fusion parents and fusion regenerants

The growth pattern as well as the growth rate of Pf67 and its protoplast regenerants from fusion process on acriflavin agar medium were shown in figure 6.5.. For the culture grow on 0, 0.001 and 0.01 mg/ml acriflavin agar medium, the mycelial growth started after one day incubation. However, on 0.1 mg/ml acriflavin agar medium, all cultures showed their measurable mycelial growth on day 3 or day 4. Complete growth inhibition for all four cultures was observed on the complete

agar medium with 1 mg/ml acriflavin. Statistical analysis of the growth rate of each strain on these drug media showed that the drug dosage which caused a statistically significant inhibition on the growth of the strains was 0.1 mg/ml acriflavin.

Considering figure 6.6., the growth pattern of the four strains on guaiacol agar media was quite similar as the results showed in figure 6.6.. However, the response of the four strains was rather different from that on the acriflavin agar media. Increase in guaiacol concentration from 0.01 to 0.1 μg/ml showed to delayed the start of mycelium 1 to 2 days. Further increase in medium's guaiacol concentration showed a complete inhibition on mycelial growth. For all four strains, increase of guaiacol amount in culture medium from 0 to 0.1 μg/ml caused an increase in growth rate when considering the day 3 to day 7 period although such growth rate increasing tend was not statistically significant.

Similar aspects on growth of Sc17 and it's protoplasts regenerants S1, S2 and S3 were showed on figure 6.7. and 6.8.. For the growth response on acriflavin agar media (figure 6.7.), all cultures showed to have their mycelial growth started after two incubation days excepted those inoculated on complete medium with 1 mg/ml acriflavin. The growth pattern for the 0, 0.001, and 0.01 mg/ml acriflavin culture showed to be rather similar. However, the colony diameters of the cultures on 0.1 mg/ml acriflavin agar media were always approximately half to that of the former three cultures. Moreover, the growth rate of the cultures on the 0.1 mg/ml acriflavin agar media showed to be significantly decreased. Acriflavin level at 1 mg/ml in complete medium showed to be able to completely inhibited the mycelial growth of the four strains. For guaiacol agar media (figure 6.8.), cultures on complete agar medium with 0, 0.01 and 0.1 μg/ml drug showed to be not much affected on both aspects of growth patterns and growth rates. However, increase in drug

concentration to 1 μg/ml guaiacol showed to be able to partially inhibit the mycelial growth such that the colony diameters of the cultures were only 1/5 of those on the complete medium without addition of guaiacol. On the other hand, the mean growth rate of the culture on the 1 μg/ml guaiacol agar media were showed to be significantly lower than that of those grow on agar media with lower level of guaiacol. Complete growth inhibition was occured on the media with 10 μg/ml guaiacol.

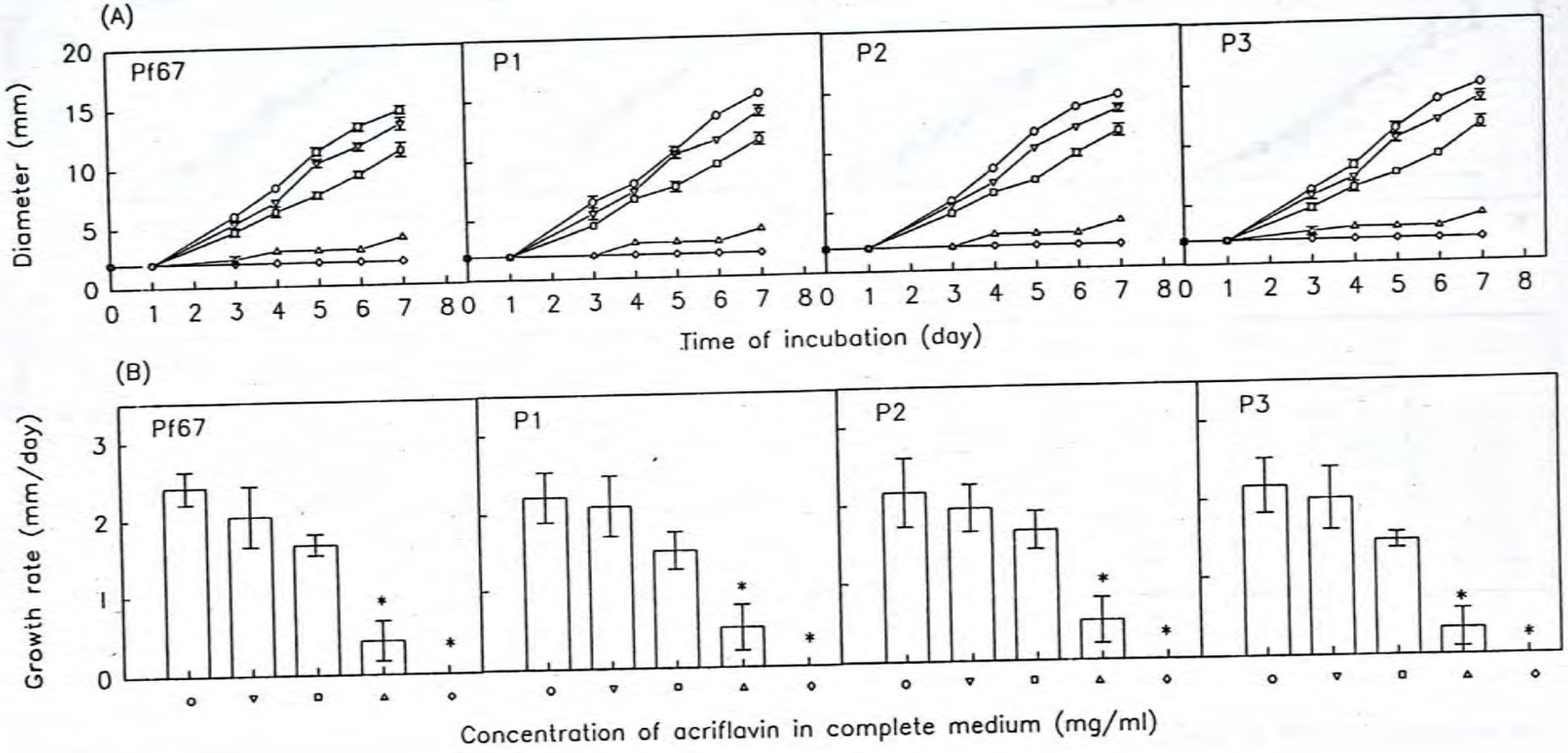


Figure 6.5. Growth curves (A) and growth rate (B) of *Pleurotus florida* Pf67, and regenerants of Pf67's protoplasts P1, P2 and P3. The three regenerants were collected after PEG treatment of Pf67's protoplasts under the same condition as descrided for fusion of Pf67 and Sc17. All cultures were kept at 28'C in darkness on potato dextrose agar (PDA) plates. Tests were carried out on complete media with different concentrations of acriflavin. For chart (A), all data points are the mean values of triplicates. For the growth rate bar charts (B), the data are the mean values of the growth rate from day 3 to day 7. For chart (A) and (B), the concentrations of acriflavin in complete media are ∘ 0 mg/ml, ▿ 0.001mg/ml, ▫ 0.01 mg/ml, ▵ 0.1 mg/ml, ◇ 1 mg/ml. * – indicated the corresponding groups are statically different from the group without the '*' sign under independent t–test with P=0.05 level. I–I is the standard error bar.

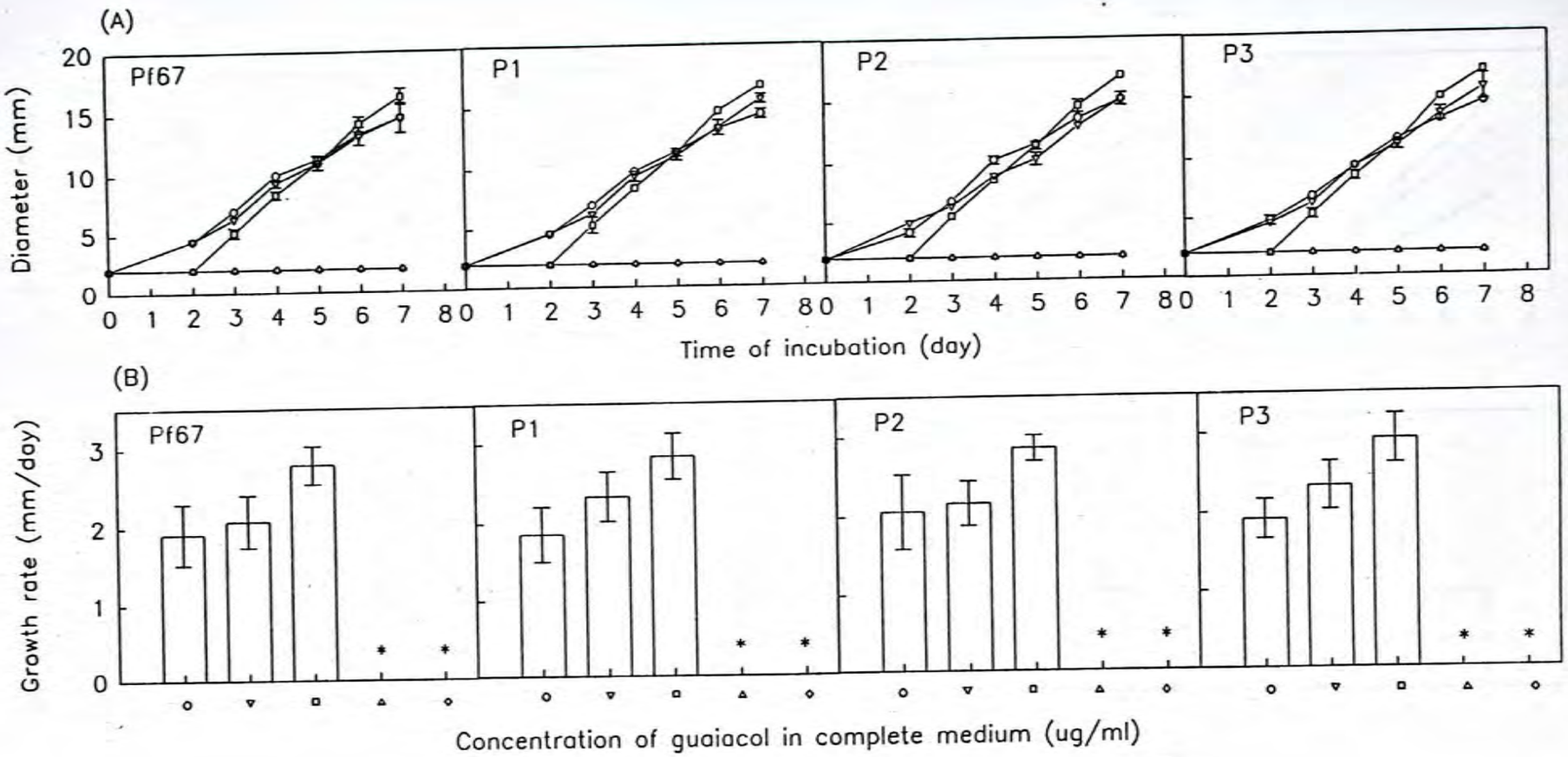


Figure 6.6. Growth curves (A) and growth rate (B) of *Pleurotus florida* Pf67, and regenerants of Pf67's protoplasts P1, P2 and P3. The three regenerants were collected after PEG treatment of Pf67's protoplasts under the same condition as descrided for fusion of Pf67 and Sc17. All cultures were kept at 28°C in darkness on potato dextrose agar (PDA) plates. Tests were carried out on complete media with different concentrations of guaiacol. For chart (A), all data points are the mean values of triplicates. For the growth rate bar charts (B), the data are the mean values of the growth rate from day 3 to day 7. For chart (A) and (B), the concentrations of guaiacol in complete media are ∘ 0 ug/ml, ▿ 0.01 ug/ml, ▫ 0.1 ug/ml, △ 1 ug/ml, ◇ 10 ug/ml. * – indicated the corresponding groups are statistically different from the the group without the '*' sign under independent t–test with P=0.05 level. I–I is the standard error bar.

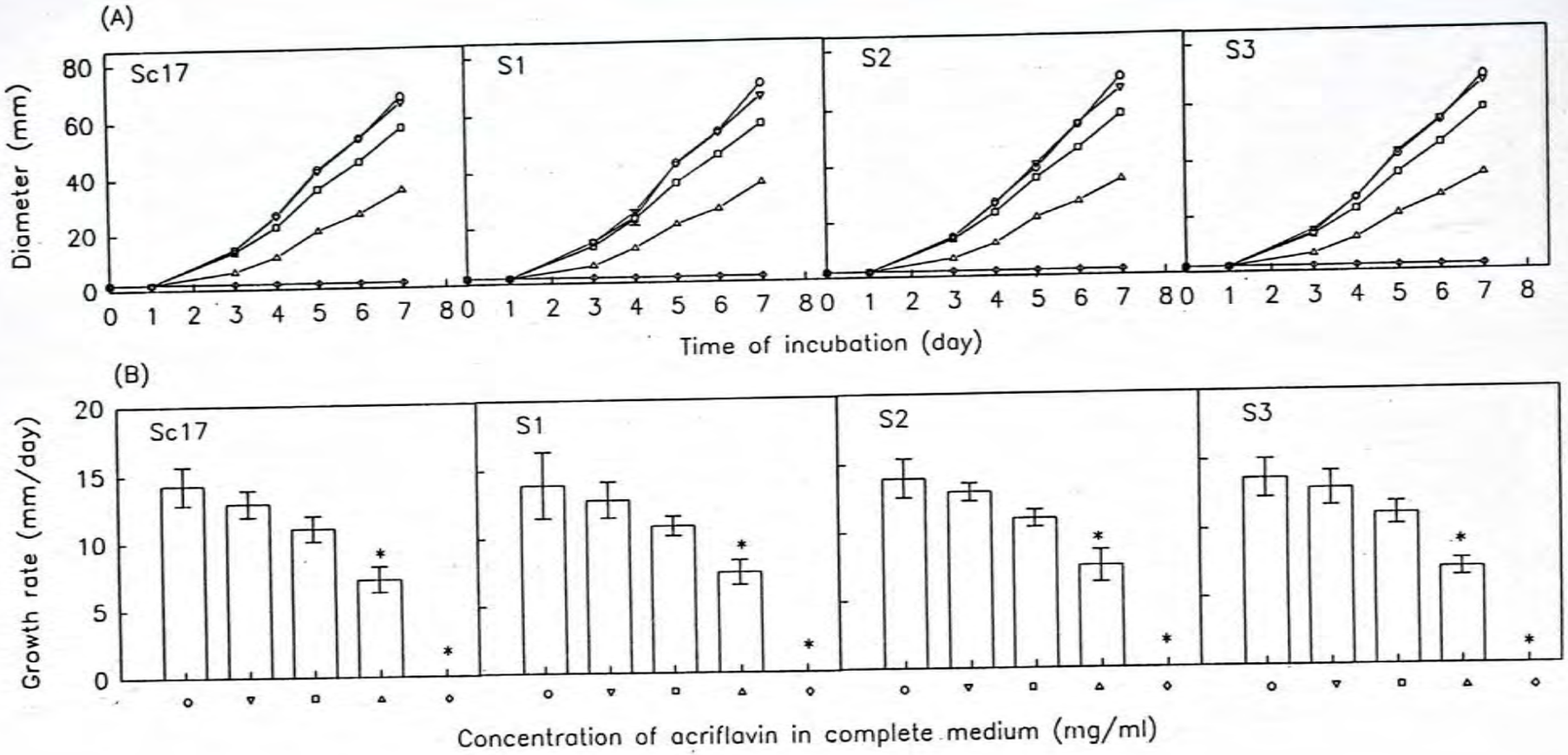


Figure 6.7. Growth curves (A) and growth rate (B) of *Schizophyllum commune*. Sc17, and regenerants of Sc17's protoplasts S1, S2 and S3. The three regenerants were collected after PEG treatment of Sc17's protoplasts under the same condition as descrided for fusion of Pf67 and Sc17. All cultures were kept at 28'C in darkness on potato dextrose agar (PDA) plates. Tests were carried out on complete media with different concentrations of acriflavin. For chart (A), all data points are the mean values of triplicates. For the growth rate bar charts (B), the data are the mean values of the growth rate from day 3 to day 7. For chart (A) and (B), the concentrations of acriflavin in complete media are ∘ 0 mg/ml, ▿ 0.001 mg/ml, ▫ 0.01 mg/ml, ▵ 0.1 mg/ml, ◇ 1 mg/ml. * – indicated the corresponding groups are statically different from the group without the '*' sign under independent t–test with P=0.05 level. I–I is the standard error bar.

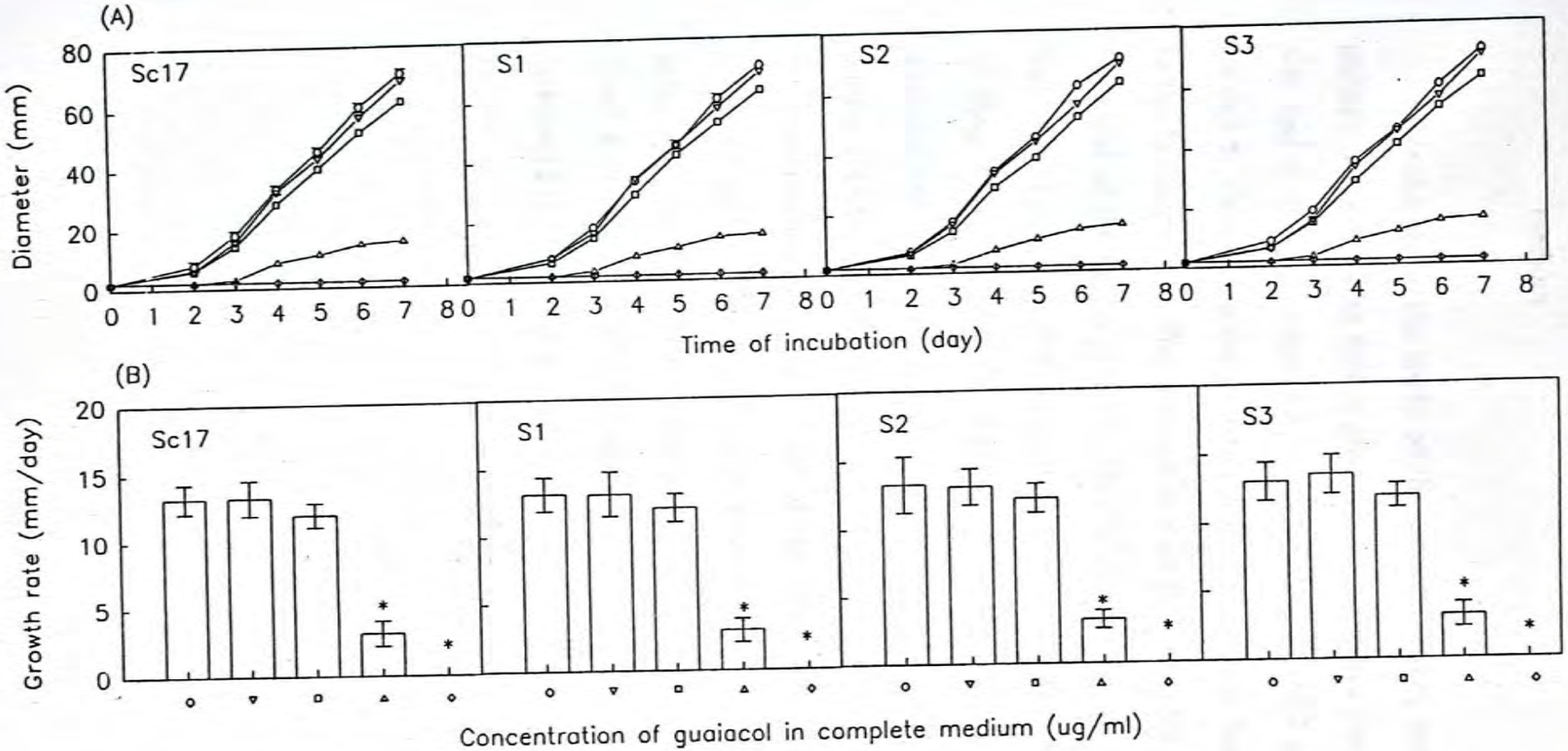


Figure 6.8. Growth curves (A) and growth rate (B) of *Schizophyllum commune* Sc17, and regenerants of Sc17's protoplasts S!, S2 and S3. The three regenerants were collected after PEG treatment of Sc17's protoplasts under the same condition as descrided for fusion of Pf67 and Sc17. All cultures were kept at 28'C in darkness on potato dextrose agar (PDA) plates. Tests were carried out on complete media with different concentrations of guaiacol. For chart (A), all data points are the mean values of triplicates. For the growth rate bar charts (B), the data are the mean values of the growth rate from day 3 to day 7. For chart (A) and (B), the concentrations of guaiacol in complete media are ∘ 0 ug/ml, ▿ 0.01 ug/ml, ▫ 0.1 ug/ml, △ 1 ug/ml, ◇ 10 ug/ml.. * – indicated the corresponding groups are statistically different from the group without the '*' sign under independent t–test with P=0.05 level. I–I is the standard error bar.

6.3.2. Growth responses of fusion parents and fusion products on complete medium

Results about the study of the growth pattern and growth rate of the fusion parents as well as the fusion products on complete medium were shown on figure 6.9. and 6.10.. From figure 6.9., both Sc17 and Pf67 started their mycelial growth on day 2. Comparing the growth patterns of the two fusion products, PS1 and PS2, to the fusion parents, they showed to start their mycelial growth at least 1 day earlier than that of the fusion parents. On the other hand, the colony diameters of the two fusion products were always greater than that of the two parents through the 7 days of incubation. Comparing the growth patterns of the PS1 and PS2, the colony diameters of the two strains were always similar. For PS3, it showed to have the colony diameters greater than that of Pf67 but only about 1/3 of that of Sc17 on day 7. Considering the growth rate of the five strains (figure 6.10.), they could be classified by t-test into three statistically different groups. The slowest growth rate group (a) was Pf67. The second group (b) had the fastest growth rate and consist of Sc17, PS1 and PS2. The last group (c) showed to have an intermediate growth rate relative to the first two groups and consist of PS3 only.

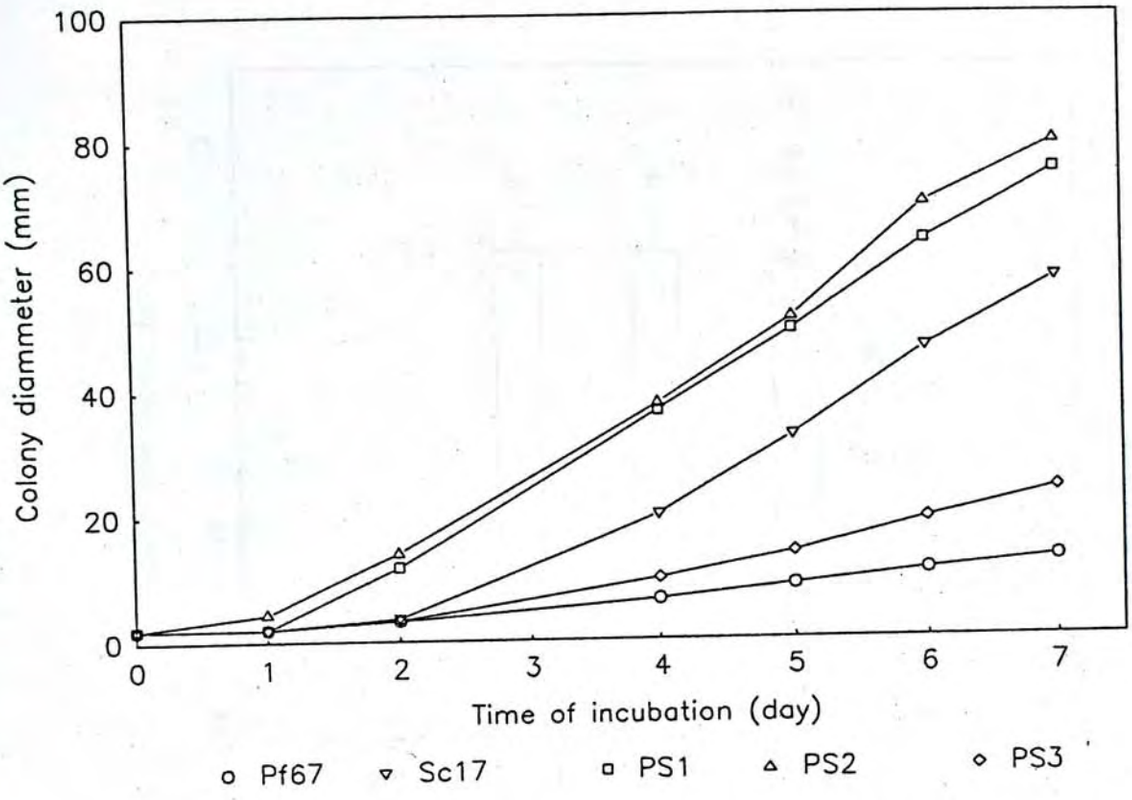


Figure 6.9. Growth curve of Pleurotus florida Pf67 and Schizophyllum commune Sc17 as well as three regenerants from fusion experiments of Pf67 and Sc17 which are PS1, PS2 and PS3 respectively. The culture medium used was complete medium agar and all cultures were kept at 25'C in darkness. All data points were the mean values of triplicates.

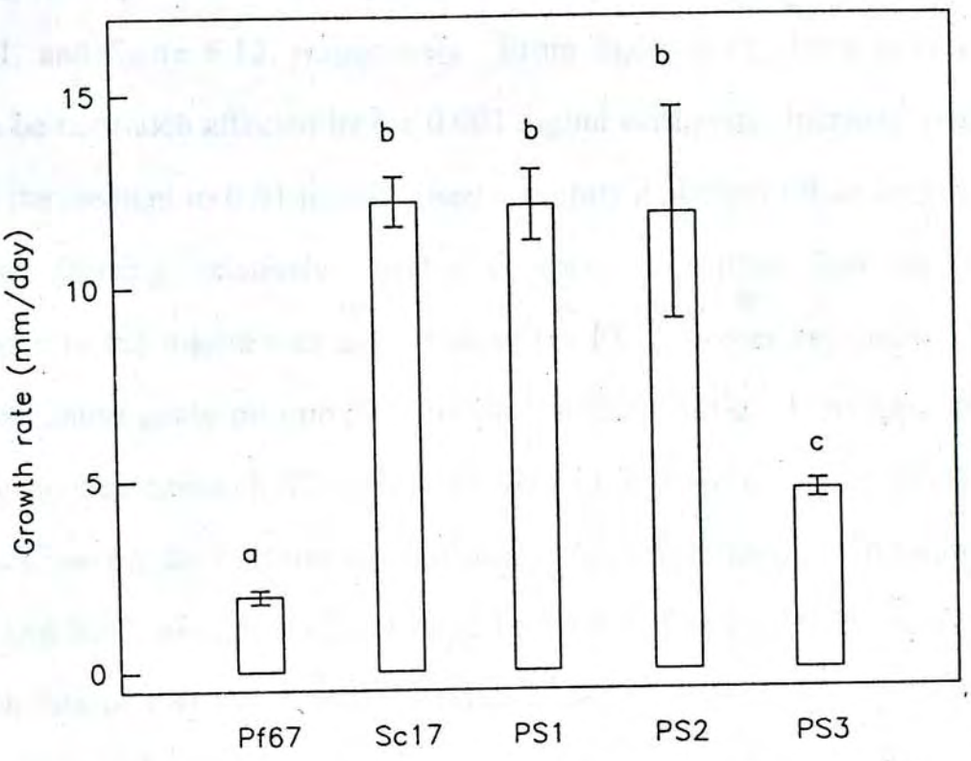


Figure 6.10. Growth rate of five different strains. Pf67 – *Pleurotus florida*, Sc17 – *Schizophyllum commune*. PS1, PS2 and PS3 are the three regenerants from the fusion experiments of Pf67 and Sc17. Growth rate is defined as the mean value of increase in colony diameter per day through day 3 to day 7. 'a', 'b' and 'c' mean that the corresponding results are belonging to three statistically different groups respectively.I–I is the standard error bar. The t–test was calculated at P=0.05 level.

6.3.3. Growth responses of fusion parents and fusion regenerants on complete medium with acriflavin

The growth pattern and the growth rate of the five strains were recorded on figure 6.11. and figure 6.12. respectively. From figure 6.11., both Sc17 and PS1 showed to be not much affected by the 0.001 mg/ml acriflavin. Increase in acriflavin amount in the medium to 0.01 mg/ml posed a slightly inhibitory effect on growth and resulted in forming relatively smaller colonies. Further increase in drug concentration to 0.1 mg/ml was able to cause the PS1 colonies approximately 30 % smaller than those grow on complete medium without drug. However, under the same drug concentration, Sc17 colony showed to be approximately 50 % smaller than those grew on the medium without drug. Although these results showed that both PS1 and Sc17 were partially inhibited by acriflavin at 0.1 mg/ml concentration, the growth rate of PS1 under this condition was not significantly decreased by the present of drug. However, Sc17 showed to be more "sensitive" and resulted in a significant decrease in mean colony diameter under 0.1 mg/ml acriflavin concentration (figure 6.12.). For both PS2 and PS3, their growth patterns showed to be not affected by the present of acriflavin up to the 0.1 mg/ml concentration (figure 6.11. and 6.12.). At 1 mg/ml acriflavin, the growth of the strain was completely inhibited as all five strains (figure 6.11. and figure 6.12.). However, the PS2 has the growth pattern rather similar to that of PS1 but PS1 showed to be much more sensitive to acriflavin. In addition, the growth rate of all three fusion products showed to be significantly reduced at 1 mg/ml acriflavin level but both fusion parents had their growth rate being reduced significantly at 0.1 mg/ml acriflavin level.

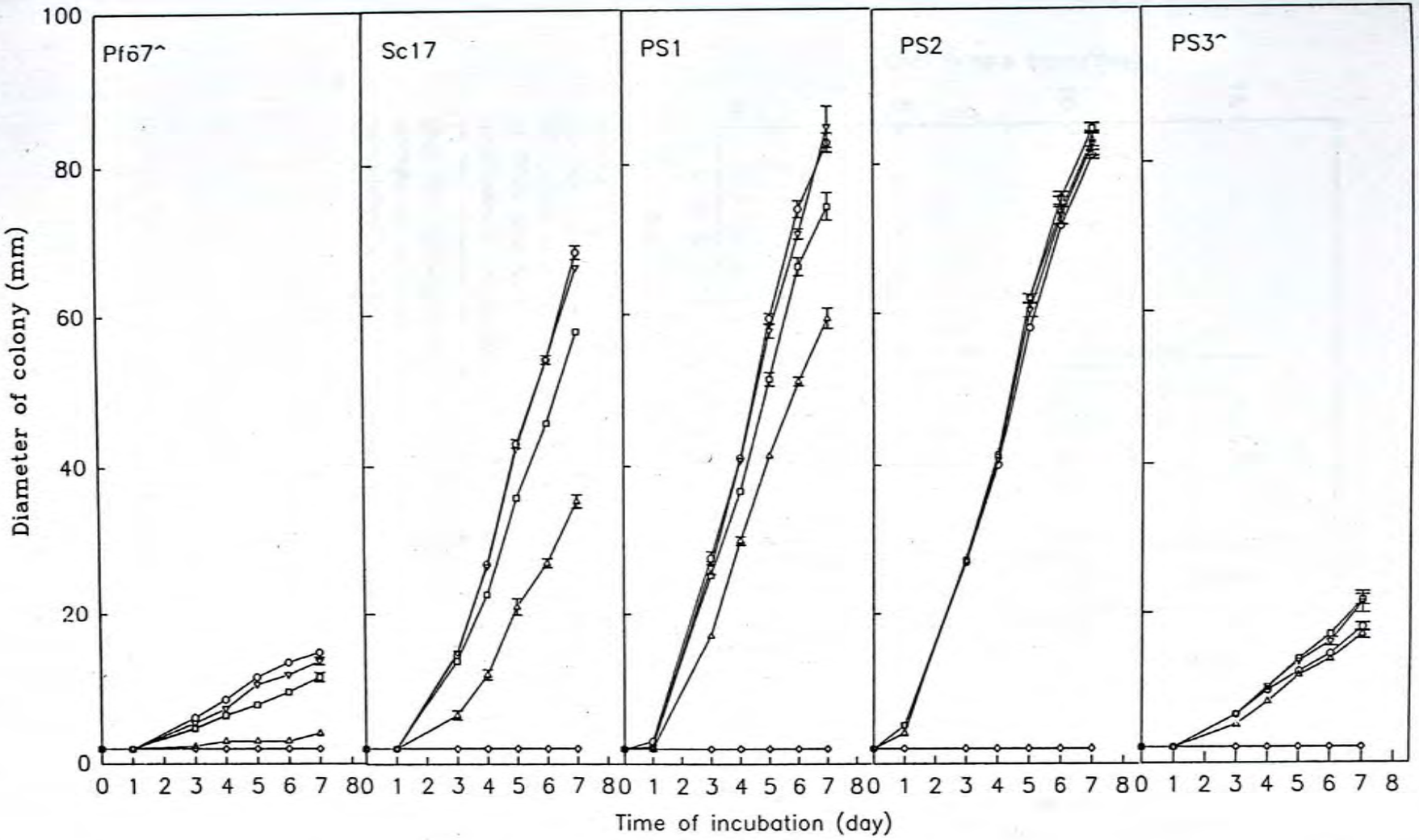


Figure 6.11. Growth curves of *Pleurotus florida* Pf67, *Schizophyllum commune* Sc17, and three regenerants from fusion experiments of Pf67 and Sc17. The three regenerants are PS1, PS2 and PS3. All cultures were kept at 25°C in darkness on pototo dextrose agar (PDA) plates. Tests were carried out on complete media with different concentrations of acriflavin. All data are the mean values of triplicates. The concentrations of acriflavin in complete media are ∘ 0 ug/ml, ▿ 0.001 mg/ml, ▫ 0.01 mg/ml, △ 0.1 mg/ml, ◇ 1 mg/ml. I–I is the standard error bar. The "^" sign indicated that the corresponding cultures showed brown staining character on all guaiacol plates. "*" sign indicated that the corresponding plate has the colony showed brown staining character.

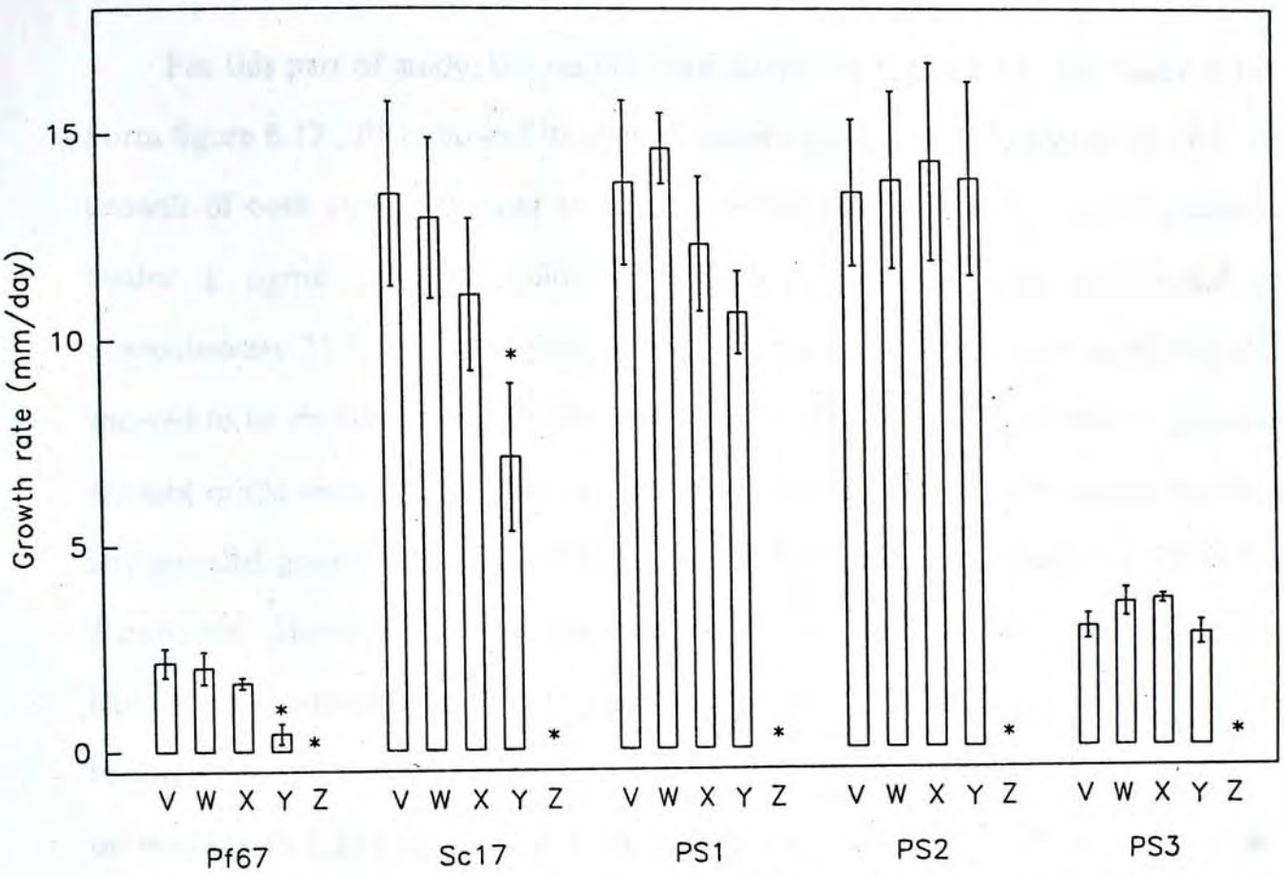


Figure 6.12. Growth rate of different strains on complete media with different concentrations of acfiflavin. Pf67 is the *P. florida*, Sc17 is the *S. commune*. PS1, PS2 and PS3 are the three regenerants collected from the fusion experiments of Pf67 and Sc17. V, W, X, Y and Z stand for 0, 0.001, 0.01, 0.1 and 10 mg/ml acriflavin in complete media respectively. Growth rate is defined as the mean values of the increase of colony diameter per day from day 3 to day 7. "*" means that that the corresponding value shows to have a statistically significant different value compared to those without the sign. I–I is the standard error bar. The t–test is calculated at P=0.05 level.

6.3.4. Growth responses of fusion parents and fusion products on complete medium with guaiacol

For this part of study, the results were shown on figure 6.13. and figure 6.14.. Form figure 6.13., PS1 showed its growth pattern quite similar to that of Sc17. The growth of both strains showed to be slightly inhibited under 0.1 μg/ml guaiacol. Under 1 μg/ml guaiacol, colonies of both strains had their size equal to approximately 25 % of those grew on media without guaiacol. Although PS2 also showed to be slightly inhibited under 0.1 μg/ml guaiacol, further increase in guaiacol amount in the complete medium to 1 μg/ml caused the strain to be unable to show any mycelial growth on the first 4 days of incubation and only slow growth from day 5 onwards. However, on the aspect of growth rate, both PS1 and PS2 showed to have their growth rate significantly reduced at 1 μg/ml guaiacol level that was similar to that of Sc17 (Figure 6.14.). Like Pf67, growth of PS3 was completely inhibited on media with 1 and 10 μg/ml guaiacol. However, unlike Pf67, the colony diameter showed to be the largest for the cultures grew on the medium with 0.1 μg/ml guaiacol. At this guaiacol, Pf67 showed a 2 days delay in starting the mycelial growth and the mean colony diameter on day seven was not significantly different from that of 0 μg/ml guaiacol (figure 6.13.). Actually, the mean growth rate of PS3 for the cultures on 0.1 μg/ml guaiacol agar media, was significantly higher than that of the others and this phenomenon did not occur in Pf67 (figure 6.14.).

For the guaiacol agar staining property of the five strains, both Pf67 and PS3 were able to stain the agar brown in the presence of guaiacol. However, agar staining phonomenon was not commonly occurred in all PS1 cultures. On the day 6 and day 7 PS1 cultures on 0.1 μg/ml guaiacol agar medium showed to stain agar brown. For PS2, brown agar staining occurred in the cultures grown on 0.1 and 1 μ

g/ml guaiacol agar from the third incubation day and the forth incubation day respectively.

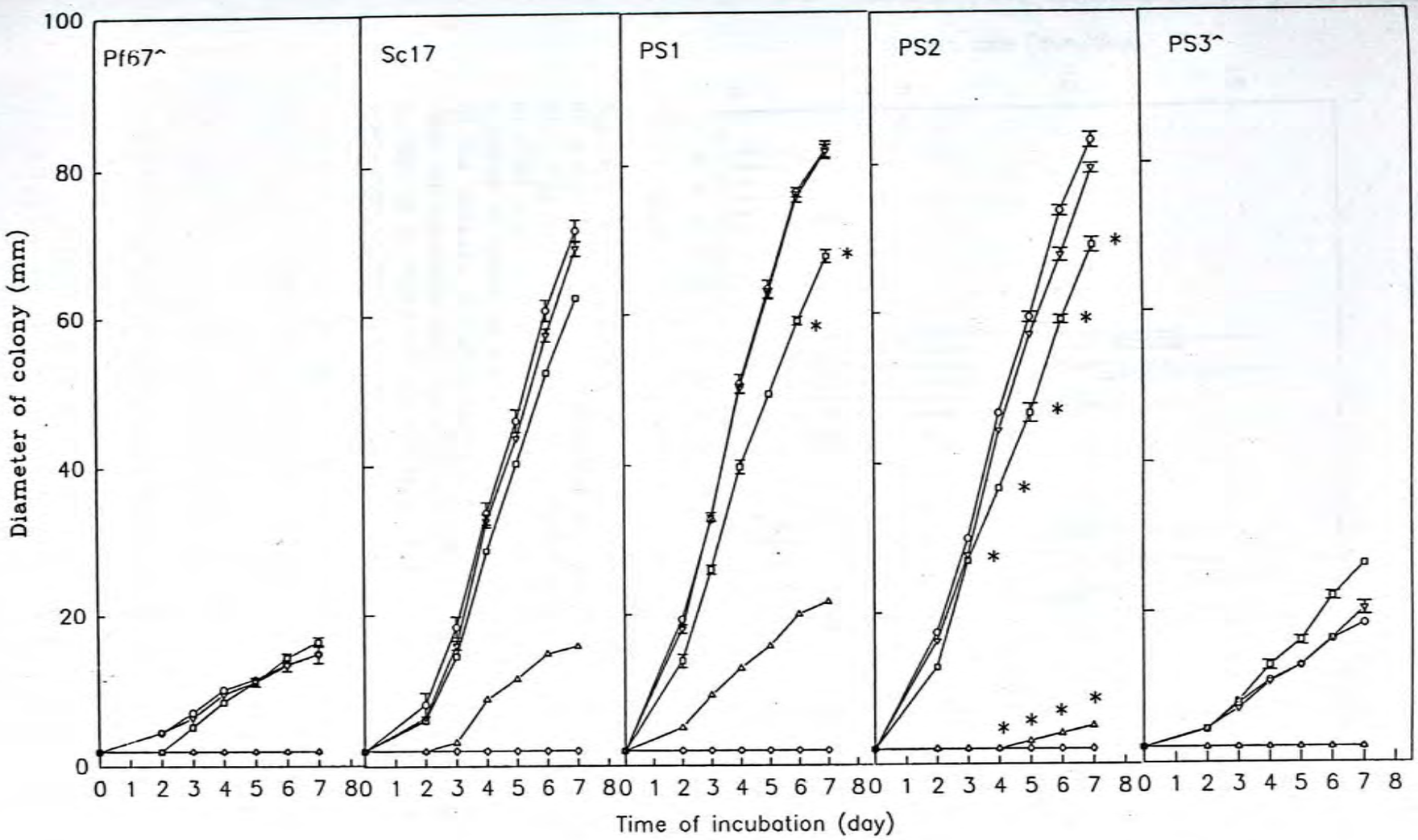


Figure 6.13. Growth curves of *Pleurotus florida* Pf67, *Schizophyllum commune* Sc17, and three regenerants from fusion experiments of Pf67 and Sc17. The three regenerants are PS1, PS2 and PS3. All cultures were kept at 25'C in darkness on pototo dextrose agar (PDA) plates. Tests were carried out on complete media with different concentrations of guaiacol. All data are the mean values of triplicates. The concentrations of guaiacol in complete media are ∘ 0 ug/ml, ▿ 0.01 ug/ml, ▫ 0.1 ug/ml, ▵ 1 ug/ml, ∘ 10 ug/ml. I–I is the standard error bar. The "^" sign indicated that the corresponding cultures showed brown staining character on all guaiacol plates. "*" sign indicated that the corresponding plate have the culture showed brown staining character.

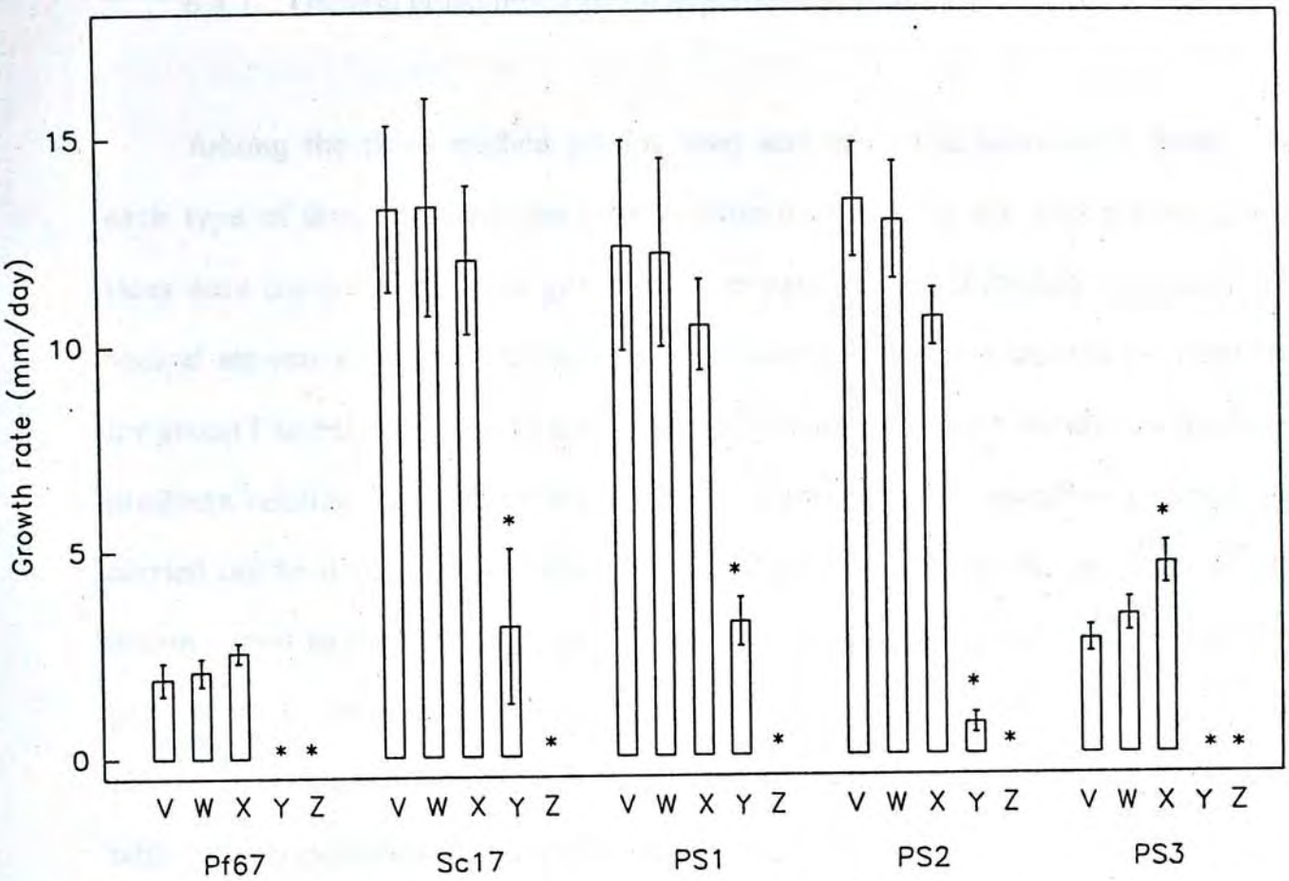


Figure 6.14. Growth rate of different strains on complete media with different of guaiacol. Pf67 is the *Pleurotus florida*, Sc17 is the *Schizophyllum commune* PS1, PS2 and PS3 are the three regenerants collected from the fusion experiments of Pf67 and Sc17. V, W, X, Y and Z stand for 0, 0.01, 0.1, 1 and 10 ug/ml guaiacol in complete media respectively. Growth rate is defined as the mean values of the increase of colony diameter per day from day 3 to day 7. "*" means that that the corresponding value shows to have a statistically significant different value compared to those without the sign. I–I is the standard error bar. The t–test is calculated at P=0.05 level.

6.4. Discussions

6.4.1. General considerations on experimental design

Among the three studied groups, two sets of comparisons were made. For each type of drug medium, the growth pattern as well as the exponential growth rates were compared between group I (fusion parents) and II (fusion products). The second set was a control experiment that concerned the same aspects as mentioned for group I and II. In order to account for any relative changes occured in the fusion products relative to those of the two fusion parents, the control experiment was carried out to determine the effect of PEG fusion process on the response of each fusion parent to the two fungitoxic drugs. That was, protoplasts from either fusion parent strain were released and subjected to PEG fusion process and the physiological response of the regenerants to the two fungitoxic drug was compared with the corresponding fusion parent strain.

Considering the aspect of the time course of the experiment, it was set to be seven days. This setting was mainly based on the limitations of the size of petri-dish. The Strains PS1 and PS2 have the fastest growth rate so that the size of agar surface became no longer large enough to allow further increase in colony diameter after day sevens of incubation. Although other strains have a relatively slower growth rate and further increase in colony sizes after seven incubation days were possible, increase in colony diameter of these slow growth strains from day 8 or later could not be compared with that of PS1 and PS2 as colonies already reached the plates margin. Therefore, assessments on the growth of the slow growth cultures on day 8 or later were unnecessary.

For the aspects of the measurement of the colony diameter, it was much more difficult for the slow growth strains especially the Pf67 and PS3. As the mycelial growth per day was only a few millimeters, any mistakes made in the measurement would affect the resulting mean colony diameter a lot. Fortunately, the growth rate of the three replicas of the these strains showed to be rather consistent. Therefore, the summation effect of technical error as well as the natural occurring variation in mycelial growth per day did not cause a high value of standard error. However, it was found that the natural occurring variation in mycelial growth per day for the fast growing strains (Sc17, PS1 and PS2) was relatively greater than that of the slow growing strains (Pf67 and PS3). That was, the range of such variation in fast growing strains was approximately 1 to 5 mm but that of slow growing strains was approximately 1 to 3 mm.

For the calculation of mean growth rate, only the growth rate within day 3 and day 7 was considered. The reason was mainly due to the general presence of lag phase before the exponential growth of the mycelium on the agar medium. The time of this lag phase varied among the five strains as well as different types of media. Generally, 3 days was found to be the maximum length of time for such phase. Therefore, the growth rate from day 3 onward was recorded.

6.4.2. Growth responses of protoplast regenerants of either fusion parents

From figure 6.5. and figure 6.7., the growth pattern of P1, P2 and P3 showed to be rather similar to that of Pf67. Considering the results mentioned in section 6.3.1., the effective dosage that was able to cause a significant inhibition of mycelial growth (0.1 mg/ml acriflavin) and the dosage which was able to caused complete growth inhibition of the mycelium (1 mg/ml acriflavin or 1 μg/ml guaiacol) as well as

the duration of lag phase of all three regenerants P1, P2 and P3 showed to be not statistically different from that of Pf67. Therefore, although non-statistically significant variation exit in the growth pattern as well as the growth rate among the four strains, the results of the four strains showed to be rather consistent.

Similar situations can be found for Sc17 and it's three regenerants S1, S2 and S3. The effective acriflavin concentration that causes significant growth inhibition of all four strains was 0.1 mg/ml and that of guaiacol was 1 μg/ml. The duration of lag phase of mycelial growth of the four strains on 1 μg/ml guaiacol was 3 incubation days. Complete inhibition of the four strains required the presence of 1 mg/ml acriflavin or 10 μg/ml guaiacol in the agar medium (section 6.3.1.).

Therefore, the study of the effect of PEG fusion process on the response of the protoplasts regenerants of the fusion parents to the two fungitoxic drugs had a rather implicative result. Although a certain extent of the genetic heterogeneity was expected (Raper and Raper, 1964), the stability of auxotrophic markers (section 4.3.3.2.) as well as the consistence in the growth response of the protoplast regenerants of either parents to the of the two fungitoxic drug showed that the established PEG fusion process in this research was unable to cause any genetical changes, which could cause significant phenotypical variation in the physiological response to the two drugs nor altering the expression of the auxotrophic markers.

6.4.3. Growth responses on complete medium without fungitoxic drug

From the result of section 6.3.2., it was showed that there was similarity and difference in the aspects of growth pattern between the two fusion parents. Considering the lag phase, both fusion parents had the duration of lag phase of two

days. However, the two fusion parents have their corresponding growth rate belong to two different statistical groups. For the fusion products PS1 and PS2, both of them showed to have their growth rate similar to that of Sc17 but different from that of Pf67 under statistical test. On the aspect of lag phase in the growth pattern of these two fusion products, they performed differently from both fusion parents such that they had a rather short lag phase which was about one incubation day. For PS3, it showed to have a lag phase duration similar to both fusion parents but the growth rate of this fusion product was different from both fusion parents statistically and was found to be intermediate between the two parents. Therefore, considering the growth of the fusion products on complete medium, all of them performed partly similar to one or both of the parents and also showed some variations in some characters from their fusion parents.

6.4.4. Growth responses on the acriflavin agar medium

Based on growth rate (figure 6.12.), both Sc17 and Pf67 at 0.1 mg/ml acriflavin agar medium showed to be partially and significantly inhibited. However, the complete growth inhibited level of acriflavin for the two strains was 1 mg/ml. Moreover, the mean growth rates of the two fusion parents were decreased as the concentration of the drug increased. When considering the growth rate of the three fusion parents, none of them showed to have the partially and significantly inhibited acriflavin level but only have the complete growth inhibition level as their two fusion parents. For PS1, the growth rate was decreased with increasing concentration of acriflavin. Although the level of decrease in growth rate was not as significant as that of the two parents, the trend of decrease in growth rate was quite similar to that of Sc17. For PS2 and PS3, both strains showed to have their growth rate not very much affected by the increase in concentration of acriflavin. This phenomenon was

rather different from the two fusion parents. As a whole, the fusion products seem to be more resistance to acriflavin relative to the fusion parents or we might sumarize that the fusion products were less sensitive to fungicidal action of acriflavin.

6.4.5. Growth responses on guaiacol agar medium

Considering the fusion parents, the growth rate of Sc17 showed to be partially and significantly inhibited by 1 μg/ml guaiacol and being completely inhibited at the 10 μg/ml guaiacol. Pf67 showed to have no partially inhibited phenomenon but had a relatively lower complete inhibition level of 1 μg/ml guaiacol. For the fusion products, PS1 showed to be rather similar to Sc17 on both aspects of partial and complete inhibition guaiacol level. On the two aspects mentioned above, PS2 showed to have an intermediate performance relative to the two fusion parents. The reason was that at 1 μg/ml guaiacol agar medium, the growth of PS2 showed to be nearly completely inhibited. However, after a long lag phase (4 incubation days), it showed a limited growth. PS3 showed to have the complete inhibition level similar to that of Pf67. However, the mean growth rate of PS3 on 0.1 μg/ml guaiacol agar medium was promoted and such phenomenon did not occur in the two fusion parents.

On the aspect of the ability to stain the guaiacol agar brown, PS1 showed to have the lowest ability to give such brown stain. Although PS1 was a dikaryon (section 5.3.3.) and expected to have a high ability to produce extracellular laccase (De Vries, 1986), the polymerization of guaiacol or the conversion of guaiacol to quinone in the medium by PS1 was found to be not extensive. The lack of ability to cause pigmentation seems to be more Sc17 like. However, the PS2 culture showed to be able to cause such pigmentation when the concentration of guaiacol was equal

to or higher than 0.1 μg/ml. Therefore, the ability of agar staining in PS2 seems to be come from induction. Anyway, such a pigmentation causing ability of PS2 was fairly Pf67 like.

6.4.6. Summary

In summary, the fusion products showed to be generally more resistance to acriflavin relative to the fusion parents. However, the response of fusion products to guaiacol showed to have much more variation from the original character of the fusion parents.

As we know that the phenotype of any organism was a manifestation of the interaction result of its genotype and the environment. Hence, phenotypic differences expressed by the three fusion products can be caused wholly or in part by the environment. However, the control experiment about the comparison of fusion parents and their corresponding protoplasts regenerants were able to rule out the possibility of the participation of the environmental effect, which was caused by the action of the PEG fusion treatment, on the phenotypic variation among the fusion products relative to the fusion parents. Therefore, the phenotypic variation might be due to the some kind of genetical mutation process occurred after protoplast fusion of the two fusion parents.

Chapter 7

Genetical Studies

7.1. Introduction

For organisms with practical value or those used in academic studies, genetical characterization using specific genetic markers is necessary. A genetic marker is defined as an allelic difference occurring at a certain position (locus) on a chromosome. This makes it possible to follow the transmission of that locus through successive generations (Chang, S. T., personal communication). That is, both biochemical and molecular markers that discriminate among individual strains are essential for the genetical characterization of an organism. A biochemical marker is considered to be a kind of genetic marker which is dealing with the chemical nature of hereditary determinants and the manner of their action in both development and function. Actually, the usefulness of biochemical marker in various kinds of genetical research cannot be denied. Nowadays, several different kinds of biochemical markers have been used in protoplast fusion experiments, the most frequently used biochemical markers in the characterization of fusion products are those concerning the nutrition abilities (auxotrophic markers) (Iijima *et al.*, 1991; Kiyohara *et al.*, 1990; Kirimura *et al.*, 1989) as well as isoenzyme analysis (isoenzyme markers) (Martini *et al.*, 1987; Toyomasu and Mori, 1987). However, detailed characterization of the genotype essential for differentiation between fusion products and parental strains can also be based on molecular analysis.

Karyological analysis can very often reveal significant chromosomal changes such as structural re-arrangements (Karp and Bright, 1985). However, chromosomal changes cannot reveal alterations in individual genes. A precise

determination of changes in a particular gene sequence can be obtained by restriction fragment length polymorphism (RFLP) analysis (Huffman *et al.*, 1992). However, the method is limited in two main ways. Firstly, the length of time required to undertake an RFLP analysis is usually 5-6 days and secondly, the result of such an analysis is limited only to the gene sequence which is used as a probe. On the other hand, the development of the polymerase chain reaction (PCR) (Saiki *et al.*, 1985), has led to the major technical advances in molecular biology in recent years. It has also allowed the isolation of particular genes to be isolated and can be used in rapid screening and sequencing of inserts. Unfortunately, the application of PCR usually requires information to be present on the sequence of the target DNA, thereby often limiting its usefulness for genomic fingerprinting. The development of a PCR process using only a single arbitrary oligonucleotide primer, known as AP-PCR (arbitrarily primed PCR) (Welsh and McClelland, 1990), makes the amplification of random sequences in genomic DNA possible. These sequences can in many cases be used as genetic markers (William *et al.*, 1990). Actually, AP-PCR is able to generate polymorphisms and such polymorphisms are strain specific. The application of AP-PCR in strain typing of *Lentinula edodes* has been suggested (Kwan *et al.*, 1992) and its application in characterization of protoplast fusion product has been suggested by Chiu *et al.* (1993).

The principle of AP-PCR is partially similar to that of PCR method. PCR is based on the enzymatic amplification of a specific DNA fragment. The target DNA fragment is flanked by two oligonucleotide primers that hybridized to opposite strands of the target sequence. The primers are oriented with their 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with DNA polymerase result in the amplification of the segment

defined by the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other primer, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million folds in a few hours (Saiki et al., 1988). For AP-PCR, a single type of primer was used for enzymatic amplification of DNA instead of using two primers. Moreover, the sequence of the primer used is not specifically designed to hybridize with a target sequence. Instead, the primer with arbitrary sequence used in AP-PCR is randomly chosen. An arbitrarily primed fingerprint is then generated by subjecting a small amount of template DNA to PCR at relaxed stringency with the randomly selected oligonucleotide primer. Such low stringency condition is maintained only for the first two cycles. After the low stringency condition is terminated, forty (or thirty-five in some experiment) thermal cycle with high stringency condition is then followed. Therefore, the DNA fragments generated under the relaxed stringency condition act as a set of templates for the specific DNA amplification in the next forty high stringency thermal cycles. It should be noted that although the whole set of DNA generated in the first two cycles is under a relaxed stringency condition, the site of primer hybridization is not random but specific. That is, although we cannot predetermine the amplification products of the first two thermal cycles, the whole set of DNA fragments generated in the first two cycles is a specific set of products under the same stringency condition (figure 7.1.). The reproducibility of AP-PCR fingerprints (Welsh *et al.*, 1991) is the best evidence for the fact described above.

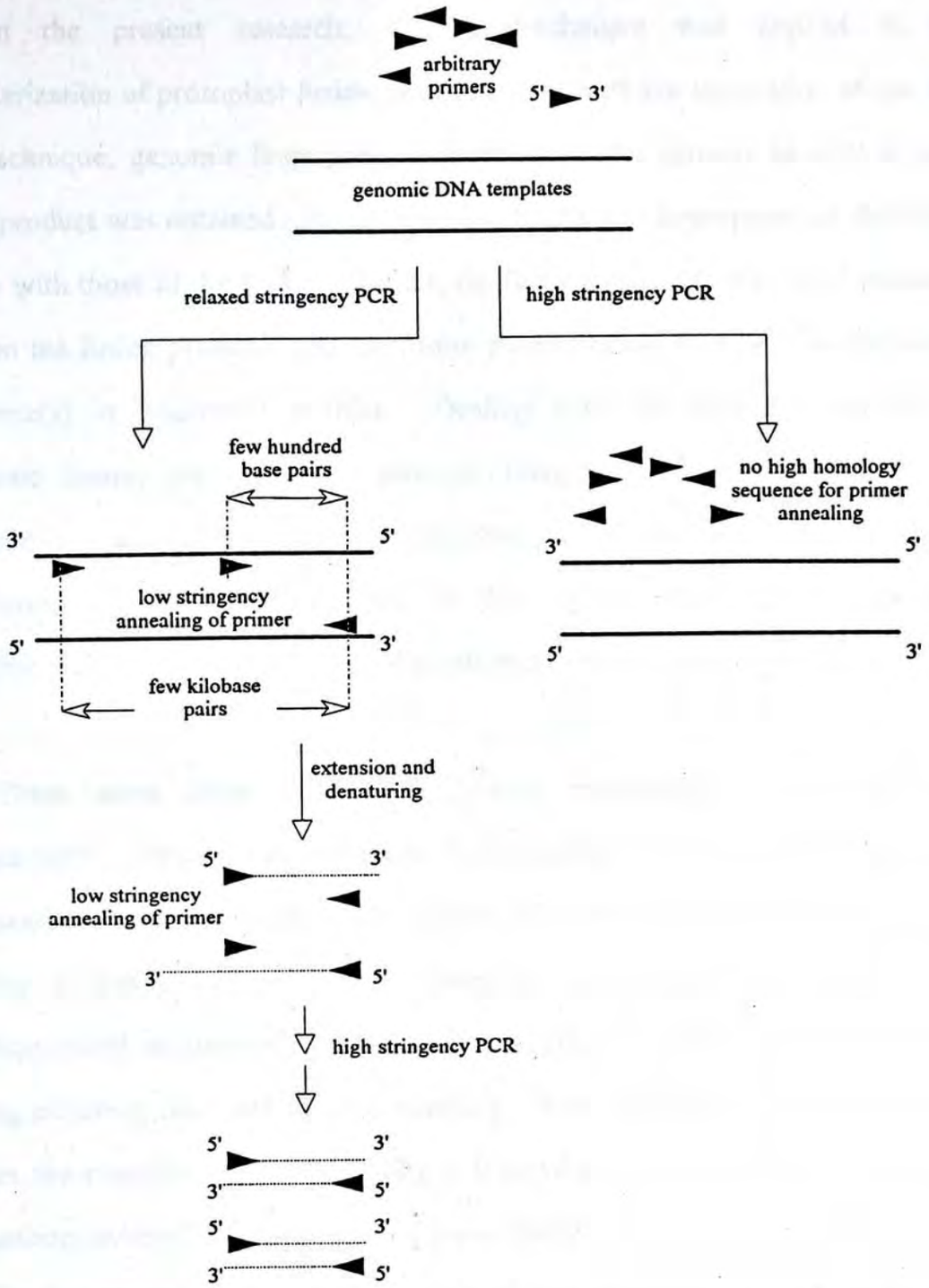


Figure 7.1. Rationale for the phenomenon of AP-PCR. At a sufficiently low temperature, primers can be expected to anneal to many sequences with a variety of mismatches. Some of these will be within a few hundred base pairs of each other and on opposite strands. Sequences between these positions will be PCR amplifiable. The extent to which sequences amplify will depend on the efficiency of priming at each pair of primer annealing sites and the efficiency of extension. At early cycles, those that prime most efficiently will predominate. At later times, those that amplify most efficiently will predominate.

In the present research, AP-PCR technique was applied in the characterization of protoplast fusion products. Through the application of the AP-PCR technique, genomic fingerprints of both the fusion parents as well as each fusion product was obtained. By comparing the genomic fingerprints of the fusion parents with those of the fusion products, the relative alternation in DNA sequence between the fusion products and the fusion parents could then be revealed as the difference(s) in fingerprint profiles. Dealing with the molecular analysis of dikaryotic fusion product PS1, genomic fingerprints of the nuclei of the corresponding two nuclear types were obtained. The two types of monokaryon of the corresponding two nuclear types in PS1 can be recovered by protoplast formation followed by regeneration (Wessels *et al.*, 1976; Yoo *et al.*, 1987).

There were other methods described previously for obtaining the monokaryotic isolates. Leal-Lara and Eger-Hummel (1982) reported to use a "monokaryotization solution", which contain only glycine and glucose, along with applying a series of mechanical damaging procedures for obtaining the monokaryotized isolates were obtained. Such method has the major disadvantages of long culturing time and labor demanding. For selecting the monokaryotized isolates, the complete process requiring at least two and a half weeks. Moreover, this method involved to repeat a lot of experimental steps such as spreading plates and blending mycelium. Increase in number of these steps would cause an increase in the chance of contamination. Actually, such method was theoretically not applicable in the present research. For one thing was that, we could not expect whether the two types of monokaryotic strains of the corresponding nuclei of PS1 were prototrophs or auxotrophs. As the monokaryotization solution only contained glycine and glucose as the sole carbon sources, only the prototrophic strains could

survive in such a medium. Therefore, such method posted a limitation in the screening process of "monokaryotization".

Other means to induce segregation of hybrids has been described by Kevei and Peberdy (1979). The "induced-haploidization" method was carried out by chemical means. Approximately 1 μg benomyl per milliliter agar medium was used for inducing segregation of inoculated mycelium. Stable haploid and unstable aneuploid isolates has been obtained by this method (Kevei and Peberdy, 1984). Although using benomyl for induced-haploidization is a conventional method for obtaining segregants, the method has the disadvantage of producing only a limited number of segregants per plate. On the aspect of the efficiency in producing a large number of segregants in one experiment, the protoplasting method should be the superior one among those methods mention above. Wessels *et al.* (1976) reported that 20-40 % of the protoplasts regenerants of the dikaryotic *Schizophyllum commune* were developed into monokaryotic mycelia.

Besides the molecular characterization of both fusion products and the two individual nuclear types of dikaryotic fusion products PS1, conventional methods of fusion products characterization by studying the auxotrophic markers and drug resistance markers of the fusion products were also carried out. In addition, hyphal confrontation experiment was carried out to distinguish the incompatibility genotype of all strains in the present fusion experiment (Darmono and Burdsall, 1992). Therefore, complementation tests were carried out among the fusion parents, fusion products and the two individual nuclear types of dikaryotic fusion products PS1.

7.2. Materials and methods

7.2.1. Segregation tests of auxotrophic and drug resistance markers in progeny of dikaryotic fusion product PS1 as well as the three fusion products.

Dikaryotic fusion product PS1 was inoculated onto PDA plate and incubated at 28 ^{o}C until the colonial mycelia reached the culture plate margin (about 7 days), stimulation for fruiting then started. Aeration of culture was increased by removing the parafilm wrap. The plate was then placed inside an incubation chamber with light dark cyle of 8 hours light and 16 hours dark (Schwalb, 1978). The plate was incubated in inverted orientation for 7 days. The environmental temperature for fruiting was 25 ^{o}C. When mature fruit body was formed with some spores landed on the cover of the petri dish to form the spore print, the fruit body was removed from the culture inside the laminar flow chamber and placed onto a 1 × 1 cm agar block with the gill side facing upwards. The bare face of the agar block was then adhere onto the inside surface of a new petri dish such that the fruit body was hanging under the inside top surface of the petri dish and the gills facing downward. The petri dish with the fruit body inside was then left overnight. Spores were expected to fall onto the inside surface of the petri dish within this period of time.

After one night, 1 ml of steril distilled water was then dropped onto the spores. The spores inside the petri dish were washed in the distilled water and collected into a sterilized one microliter microcentrifuge tube. The number of spores in suspension solution were counted by using haemocytometer and the concentration of spores was determined. The spore solution was then serial diluted by sterilized distilled water to obtain a spore solution with a concentration of 650

spores per milliliter. Fifty spores was then plated onto each petri dish. Ten petri dishs with spores were produced for each fruit body. The petri dishes were incubated at 28 ^{o}C for 48 hours the number of spores regenerated colonies per plate was counted. Colonies were then inspected under 160 × light microscope such that the colonies which were completely isolated were marked on the back of the plate. Fifty spore regenerated colonies were selected and each one were then pick up and inoculated into a 5 cm diameter PDA plate. The petri dishes were incubated at 28 ^{o}C for 3 days. Each single spore isolate was inspected under light microscope for clamp connection for confirmation of single spore origin.

Minimal medium (MM) agar plate supplemented with adenine, nicotine, adenine and nicotine as well as the one without supplementation were prepared as described in section 4.2.3.1.. Complete medium with acriflavin and guaiacol as well as the one without the addition of the drugs were also prepared as those described in section 4.2.3.1.. Preparation of MM supplemented with adenine and nicotine were also the same as those described in section 4.2.3.1.. The 1.5 mm diameter inoculum of each single spore isolate was inoculated onto the CM, MM, nutrient supplemented MM media and drug added CM media. The plates with inoculum were incubated for three days and the number of viable isolates on each type of plate was count. On the other hand, using the similar method, the regeneration frequency of the spores from wild type fruit body was also determined. The regeneration frequency of the spores from three fruit bodies of PS1 and another three fruit bodies wild type fruit body. Progeny analysis of both auxotrophic and biochemical markers of the single spore isolates was carried out for two fruit bodies of PS1.

Auxotrophic markers as well as the drug resistance markers of the three fusion products were also determined by the same method for the progenies of PS1 mentioned above.

7.2.2. Complementation test of fusion products as well as the spore germinants of dikaryotic fusion product PS1

This section of experiment consisted of two main parts. The first part was the complementation test between the fusion parents and the fusion products. The second part was the complementation test between the spore germinants of the dikaryotic fusion product PS1.

For the first part of study, pairs of complementation tests were carried out as table 7.1.. The M4-1 and M9-1 were used as the representative of the two individual nuclear groups of the dikaryotic fusion products.

Table 7.1. Complementation tests of fusion parents and fusion parents as well as the two nuclear type of the dikaryotic fusion product PS1.

	Pf67	Sc17	PS1	PS2	PS3	M4-1	M9-1
Pf67	N. D.*	×	×	×	×	×	×
Sc17	×*	N. D.*	×	×	×	×	×
Pf 4	×	×	×	×	×	×	×
Sc 4	×	×	×	×	×	×	×

*Remarks : N. D. - Not determined, × - test carried out.

For the second part of this experiment section, forty single spore isolates per fruit body of dikaryotic fusion product PS1 from each of the three PS1 fruit bodies were collected as described in section 7.2.1.. For each fruit body, the forty single spore isolates were randomly divided into 4 groups. Each group consist of 10 single spore isolates. Complementation test was carried out among the ten isolates of each group. Therefore, 180 mating tests were carried out for each fruit body and totally 540 complementation reaction were inspected for the three fruit body. Complementation test was carried out by inoculating a 1.5 millimeter diameter inoculum of one isolate into a 5 centimeters PDA plate and then another same size inoculum of the pairing isolate was then inoculated 3 millimeters apart from the first one. The plates were then incubated at 28 ^{o}C for three days. After incubation, the mycelia from the interacting zone of the mating pair were inspected under a 400 × light microscope. The presence of clamp connection was used as criteria for positive complementation reaction (+). Mating pairs with barrage in the interacting zone as well as rich aerial hyphae was identified to be a common B reaction (B). However, when the mating pair have clear confrontation line as well as rare aerial hyphae, it was identified as a common A reaction (F). When there was no observable interaction between the mating pair, it was identified as non-complemented reaction (-) (Chang and Lui, 1969).

7.2.3. Recovery of the individual nuclear type of dikaryotic fusion product PS1

The fusion products PS1 from the fusion experiment of Pf67 and Sc17 was maintained on PDA medium at 28 ^{o}C. Characterization of the karyotic stage by fluorescent staining of nuclei using DAPI in chapter 5 identified the strain to be a dikaryon.

Four days cultures of PS1 on PDA were collected. Mycelial mat of one culture plate was collected by scraping with the use of inoculation knife. The collected mycelia were then placed into sterilized waring blender. Twenty milliliters of sterilized distilled water was added to the mycelia inside the blender. The mixture was then blended for 25 seconds twice. Three milliliters of the blended mixture was inoculated into 20 ml of sterilized MYG liquid medium. The culture was then incubated at 28°C for 15 hours without shaking. After incubation, mycelia were then collected by centrifugation. For collecting mycelia, the culture was transferred into a sterilized centrifuge tube and centrifuged at 1,400 g for 10 minutes by Hettich EBH 3S table top centrifuge. The supernatant was discarded and the mycelial pellet was used for protoplast release.

The enzymatic condition for protoplast release of PS1 was similar to that of Sc17. Eighteen milligrams of Lywallzyme and five milligrams of Novozyme234 were dissolved into each milliliter of 0.8 M mannitol solution with 0.05 M Sorenzen's phosphate buffer at pH 5.8. Two milliliters of enzyme solution was sterilized by filtration through millipore filter with 0.25 μm pore size. Approximately 0.2 mg wet weight of PS1 mycelia was resuspended into the enzyme solution. The mixture was then incubated for 150 minutes at 32°C.

After protoplast was released in the enzyme solution, protoplasts were then separated from the undigested cell debris by filtration. Approximately 2 cm thick cotton wool was placed inside a plastic syringe and then sterilized by autoclaving at 121°C for 15 minutes. After the syringe was cooled to room temperature, the enzyme solution containing protoplasts and undigested cell debris was poured into the autoclaved syringe and press through the cotton wool column. The protoplasts solution was then collected as filtrate. The protoplasts was then separated from the

enzyme solution to prevent excessive enzymatic reaction on the protoplasts. The protoplasts was collected as a pellet by centrifugation at 1,400 g for 10 minutes and the supernatant was discarded. For washing the protoplasts to remove the enzyme solution completely, the collected protoplasts was then resuspended into 2 ml of 0.8 M mannitol solution and centrifuge again twice. Finally, a protoplasts solution of PS1 with 10^5 protoplasts per ml was obtained.

For regeneration of PS1 protoplasts, 0.2 ml of the protoplasts solution was spreaded onto each RCM and incubated for 2 days at 28 ^{o}C. Regenerated colonies on each RCM plated were inspected for the presence of clamp connection under a 400 X phase-contrast light microscope [Zeiss]. Those colonies without clamp connection on mycelium were subculture onto individual PDA plate and incubated at 28 ^{o}C for 5 days. Each subculture was further inspected for the presence of clamp connection to confirmed the monokaryotic identity. All monokaryon cultures collected was named with "M" as the first letter to indicate its monokaryotic nature. A numerical number was also given after "M" and such number "×" is assigned according to their chronological order of subculturing from the RCM plates.

The regenerated M× monokaryotic regenerants was then further characterized into two groups which belongs to the respective two nuclear types of PS1. Mating test was then carried out for the above purpose. All mating reaction was carried out on PDA medium plates. After inoculation of the two strains 1 cm apart onto each PDA medium for crossing, all reaction plates was incubated at 28^{o}C for 5 days. The mycelia along the confrontation zone were investigated for the presence of clamp connection using the 400 × phase-contrast light microscope [Zeiss]. Compatible or positive mating reaction was identified by the presence of

true clamp connection and non-compatible or negative mating reaction was identified by the absence of clamp connection. Tester strain was obtained by arbitrary choosing one M× strain and crossing with another five strains. Through this incompatibility test, another M×' strain, which showed to have positive mating reaction with the M× strain, was obtained as the second tester strain. Then, mating test of the other twenty-nine M× strains was then carried out with the two tester strains. On the other hand, all the twenty nine M× strains were also crossed with Sc17 and Pf67.

7.2.4. Genomic fingerprinting

7.2.4.1. Strains and culture medium

Twelve fungal strains were used for extraction of genomic DNA. Their sources are listed in table 7.2.. Cultures were maintained on PDA medium at 28 °C. Mycelia cultured for genomic DNA extraction were cultured in PDB medium, 24g/L. All liquid cultures were incubated at 28 °C for three to five days in darkness without shaking (depended on the growth rate of the strain).

Table 7.2. Twelve fungal strains used for extraction of genomic DNA.

Strains	Sources	Incubation time of mycelium preparation used for genomic DNA extraction
Pf67	Single spore isolate provided by Professor Chang S. T.	7 days
Sc17	Single spore isolate provided by Professor Chang S. T.	4 days
PS1	Fusion product of Pf67 and Sc17 from section 4.2.5. (Chapter 4)	2 days
PS2	Fusion product of Pf67 and Sc17 from section 4.2.5. (Chapter 4)	7 days
PS3	Fusion product of Pf67 and Sc17 from section 4.2.5. (Chapter 4)	7days
M4-1	Monokarytic isolate from haploidization of PS1 (Chapter 7, section 7.2.4.)	4 days
M4-2	Monokarytic isolate from haploidization of PS1 (Chapter 7, section 7.2.4.)	4 days
M4-3	Monokarytic isolate from haploidization of PS1 (Chapter 7, section 7.2.4.)	4 days
M9-1	Monokarytic isolate from haploidization of PS1 (Chapter 7, section 7.2.4.)	4 days
M9-2	Monokarytic isolate from haploidization of PS1 (Chapter 7, section 7.2.4.)	4 days
M9-3	Monokarytic isolate from haploidization of PS1 (Chapter 7, section 7.2.4.)	4 days

7.2.4.2. Genomic DNA preparation by cesium chloride (CsCl) method

The method of genomic DNA preparation used was a modified procedure of Yoon *et al.* (1991). Mycelia were collected from PDB cultures by centrifugation at 2,838 g using Hettich EBH 3S table top centrifuge. About 300 mg wet weight of fresh mycelia's pellet was ground in liquid nitrogen using a precooled mortar and pestle. The ground mycelium was transferred into a 1.5 ml precooled Eppendorf microcentrifuge tube. The tube was then filled with 500 µl of lysis buffer and incubated at 65 ^{o}C for 1 hour. Seven hundred microliters of chloroform was added and the tube was being inverted gently several times. The mixture was then centrifuged at 1,1500 g using Micro Centaur table-top centrifuge for 15 minutes at room temperature. Five hundred microliters of the top phase were collected to a new 1.5 ml tube and the cell debris as well as the bottom phase solution left behind was discarded. Fifty microliters of 3M NaOAc and 550 µl of ice-cold isopropanol was added to the tube and the mixture was mixed gently by inverting the tube several times. The precipitated crude DNA was collected by centrifugation at 1,1500 g at 4 ^{o}C for 15 minutes. The DNA pellet was rinsed with ice-cold 70 % ethanol twice and then dried at room temperature in vacuum for 10 minutes. The pellet was resuspended in 100 µl TE. Ten microliters of RNAase solution was added to the DNA solution and incubated at 37 ^{o}C for 2 hours. One hundred and ten milligrams of CsCl were added to the tube and gently shake to dissolve the CsCl. The solution was then centrifuged again at 1,1500 g at 4 ^{o}C for 15 minutes. One hundred and ten microliters of the solution underneath the top pellet was collected to a new tube and the pellet was discarded. Three hundred and thirty microliters of TE and 440 µl ice-cold isopropanol were added to the tube. The precipitated DNA was then collected by centrifugation at 1,1500 g at 4 ^{o}C for 15 minutes. The pellet was then washed

with ice-cold 70 % ethanol twice and dried at room temperature in vacuum for 10 minutes. The DNA pellet was dissolved in 50 µl TE and kept at 4 °C for storage.

7.2.4.3. Genomic DNA preparation by chloroform : TE saturated phenol method

This method of genomic DNA preparation used was a modified procedure of Lee and Taylor (1990). About 300 mg wet weight of fresh mycelia's pellet was collected and grinded in liquid nitrogen as described in section 7.2.4.2. The ground mycelium was transferred into a 1.5 ml precooled Eppendorf microcentrifuge tube. The tube was then filled with 400 µl of lysis buffer and incubated at 65 °C for 1 hour. Four hundred microliters of chloroform : TE saturated phenol was added and vortex briefly. The mixture was then centrifuged at 1,1500 g using Micro Centaur table-top centrifuge for 15 minutes at room temperature. Three hundred and fifty microliters of the top phase were collected to a new 1.5 ml tube. The cell debris as well as the bottom phase solution left behind was discarded. Ten microliters of 3M NaOAc and 0.54 volumes of isopropanol was added to the tube and the mixture was mixed gently by inverting the tube several times. The precipitated crude DNA was collected by centrifugation at 1,1500 g at room temperature for 15 minutes. The supernatant was discarded and the DNA pellet was rinsed once with 70 % ethanol. The pellet was drained to dry by inverting the tube on paper towel for 1 minute. Then the pellet was dried at room temperature in vacuum for 15 minutes. The pellet was resuspended in 100 µl TE. Ten microliters of RNAase solution was added to the DNA solution and incubated at 37 °C for 2 hours. The chloroform : TE

saturated phenol extraction was repeated and the purified DNA was finally dissolved in TE with 0.1 to 1 μg/ml concentration.

7.2.4.4.Qualitative analysis of genomic DNA

To investigate the purity and the molecular weight of the genomic DNA, agarose gel electrophoresis was used. The method used was similar as the procedure of Yoon *et al.* (1991). To make a 0.8 % agarose gel, 240 mg of type 1 agarose (SIGMA) were added to 30 ml of 0.5 X TBE buffer (pH 8.0). The solution was heated to dissolve the agarose. After the agarose solution was cooled to 60 ^{o}C, the solution was poured into gel mold and allowed to solidify at 4 ^{o}C. The solidified agarose gel was then placed into the gel tank. One half diluted TBE buffer was then added into the gel tank until the gel was being covered to a depth of about 1 mm. Ten microliters of DNA were then mixed with 2 ul of loading buffer. The mixture was then loaded into the slot of the submerged gel using a disposable micropipette. After the lid of the gel tank was closed and the electrical leads were attached so that the DNA will migrate toward the anode, a DC voltage of 5 V/cm was applied. The condition was maintained unchanged until the bromophenol blue dye has migrated 2/3 length of the gel. The electric current was then turned off. The DNA in agarose gel was then stained by immersing in water containing ethidium bromide (0.5 μg/ml) for 45 minutes at room temperature. The DNA banding pattern in the gel was examined by ultraviolet light. Photograph of the DNA banding pattern was carried out using Polaroid Type 667 (ASA 3000) film.

7.2.4.5. Quantitative analysis of genomic DNA

For quantitating the amount of DNA, spectrophotometric method was used. Five microliters of DNA solution was mixed with 0.5 ml of autoclaved distilled water inside a quartz curette and the reading at 260 nm was taken using the SPECTRONIC ARRAY 3000 spectrophotometer. An A_{260} of 1 corresponds to approximately 50 μg/ml for double-stranded DNA. Therefore, amount of DNA should be equal to $A_{260} \times 50$ μg/ml × 0.5 ml × 500 μl / 5 μl = $A_{260} \times 2.5$ μg/μl.

7.2.4.6. DNA amplification by arbitrarily primed - polymerase chain reaction

For fingerprinting a fungal strain, arbitrarily primed - polymerase chain reaction was used for generating specific DNA profiles for all the strains in table 7.2.. The following method was a simple modification of the procedure of Welsh and McClelland (1990) (Wong, K. K., personal communication). Fifty microliters reaction mixture contained 2.5 units of AmpliTaq polymerase, 1 X PCR Buffer II, 0.2 mM each dNTPs, 4 mM $MgCl_2$, 10 μM primer (Operon Technologies, Alameda) and 100 to 150 ng template DNA was pipetted into a 0.5 ml microcentrifuge tube. Mineral oil was overlaid onto the mixture and the tube was placed into the Perkin Elmer Cetus DNA Thermal Cycler 480. Four different primers was used in my study. They are listed in table 7.3.. The mixture was then subjected to the following thermal cycles :

Two cycles of low stringency amplification :

94 °C, 5 minutes for denaturing template DNA ;

35 °C, 5 minutes for low stringency annealing of primer ;

72 °C, 5 minutes for primer extension.

Forty cycles of high stringency amplification then followed :

94 °C, 1 minutes ; 55 °C, 1 minutes ; 72 °C, 1 minutes.

One cycle of high stringency amplification with longer primer extension time:

94 ^{o}C, 1 minutes ; 55 ^{o}C, 1 minutes ; 72 ^{o}C, 10 minutes.

The mixture was then kept in 4 ^{o}C for further analysis. Agarose gel electrophoresis was then carried out for the mixture as described in section 7.2.5.2.. Instead of using 0.8 % agarose gel, 3 % gel was used instead. The 3 % gel was prepared by mixing 1 % synergel (DIVERSIFIED BIOTECH) with 1 % type 1 agarose (SIGMA) in 0.5 X TBE buffer with 5 % ethanol.

Table 7.3. The four arbitrarily chose primer used for fingerprinting fungal strains.

Name of primer	DNA sequence of the corresponding primer
M13 forward sequencing primer (-47)	5' d-CGCCAGGGTTTTCCCAGTCACGAC-3'
M13 reverse sequencing primer (-48)	5' d-AGCGGATAACAATTTCACACAGGA-3'
*Eco*RI-Ext primer	5' d-TAGGCGTATCACGAGGCCCT-3'
Gal K primer	5' d-TACGGTGGCGGAGCGCAGCA-3'

7.3. Results

7.3.1. Progeny analysis and determination of auxotrophic as well as drug resistance markers

The results of the investigation on germination frequency of the spores from PS1 and the wild type fruit bodies were shown in table 7.4.. Independent t-test of the mean regeneration frequency of PS1 and wild type at P = 0.05 level was carried out. The t value was found to be equal to -0.3266 and P value = 0.7631. Therefore, there was no significant different between the germination frequencies of the two strains.

Table 7.4. Germination frequency of spores from PS1 and wild type fruit bodies.

	mean germination frequencies for each fruit body			mean germination frequencies for the spores of the strain
Fruit body	1	2	3	
PS1	31.6 %	30.7 %	26.1%	29.47 %
Wild type	30.6 %	49.6 %	17.4 %	32.53 %

Results of the progeny analysis of the spore germinants of the dikaryotic fusion product PS1 was shown in table 7.5..

Table 7.5. Number of spore germinants with different types of auxotrophic and drug resistance markers.

	Number of spores	
	First fruit body	Second fruit body
Total single spore isolates obtained	50	48
ade$^+$, *nic*$^+$	18 (36%)	19 (39.6%)
ade$^-$, *nic*$^-$	4 (8%)	6 (12.5%)
nic$^-$	10 (20%)	6 (12.5%)
ade$^-$	18 (36%)	16 (33.3%)
Acriflavin sensitive (100 µg/ml)	6 (12%)	7 (14.6%)
Guaiacol sensitive (1 µg/ml)	0 (0%)	0 (0%)
Oxidize guaiacol into brown product	4 (8%)	1 (2.1%)

For the determination of auxotrophic and drug resistance properties of the fusion parents, fusion products as well as the individual nuclear type of PS1, the results were listed in table 7.6..

Table 7.6. The auxotrophic and drug resistance properties of the fusion parents, fusion products as well as the individual nuclear type of PS1.

	Fusion parents		Fusion products			Two individual nuclear types of PS1	
*Genetic markers	Pf67	Sc17	PS1	PS2	PS3	M4 type	M9 type
Auxotrophic markers	**ade$^-$**, *nic*$^+$	**ade$^-$**, ***nic*$^-$**	**ade$^-$** (leaky), *nic*$^+$	ade$^+$, *nic*$^+$	ade$^+$, *nic*$^+$	**ade$^-$**, *nic*$^+$	ade$^+$, *nic*$^+$
Drug resistance markers	**Acr^S**, **Gua^S**, **B**	Acr^r, Gua^r	Acr^r, Gua^r	Acr^r, Gua^r, **B**	Acr^r, **Gua^S**, **B**	Acr^r, Gua^r	Acr^r, Gua^r

*Remarks : The concentration of adenine and nicotine in the minimal medium for the test were 0.5 μg/ml. Concentration of acriflavin and guaiacol in the complete media were 100 μg/ml and 1 μg/ml respectively. B - the agar underneath the colony stained brown in guaiacol medium.

7.3.2. Complementation tests of the fusion products as well as the spore germinants of dikaryotic fusion product PS1

The results of the complementation test among the fusion parents, fusion products and the M4-1 and M9-1 were listed in table 7.7.

Table 7.7. Complementation tests of fusion parents and fusion parents as well as the two nuclear type of the dikaryotic fusion product PS1.

	Pf67	Sc17	PS1†	PS2	PS3	M4-1	M9-1
Pf67	N. D.*	-	-	-	-	-	-
Sc17	-*	N. D.	+	-	-	-	+
‡Pf4	+*	-	-	-	+	-	-
‡Sc4	-	+	+	-	-	+	+

* : N. D. - Not determined, + - clamp connection was being found, - - no clamp connection was being found.
† : The + results indicated the fruit body formation of the Sc17 or Sc4 colony.
‡ : Pf4 and Sc4 were the mating type strains of Pf67 and Sc17 respectively.

In the complementation reaction between Pf67 and Sc17, the mycelium of Sc17 overlapped with that of Pf67 along the interaction zone. The morphology of overlapping was just like the Sc17 mycelia "climb" onto that of Pf67 (figure 7.2.). On the other hand, there was a dusty black coloration formed inside the agar underneath the interacting zone (figure 7.3.). Both phenomena were also found in

the reactions between Pf67 or PS3 with PS1, M4-1 as well as M9-1. Microscopic observations using 200 × and 400 × phase contrast light microscope [Zeiss] found that the coloration was actually formed by a lot of black particular substances suspended inside the agar (figure 7.4. (a) and (b))..

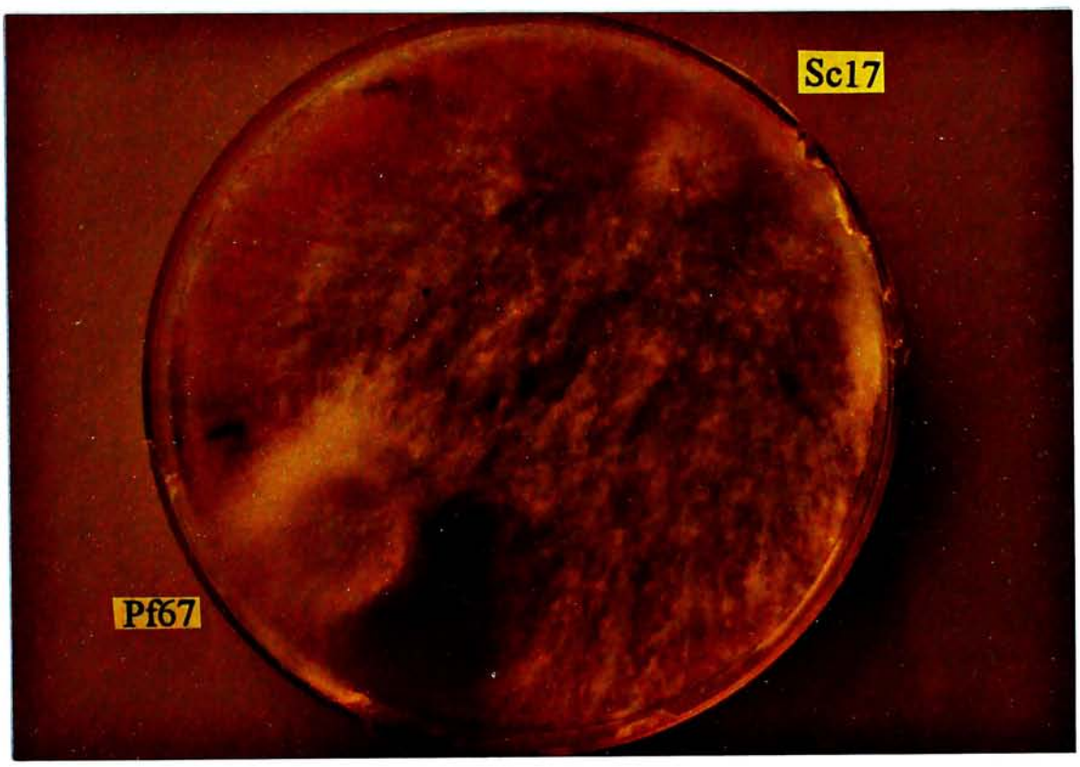


Figure 7.2. The "climbing" morphology of the mycelia along the interacting zone of the complementation test between Pf67 and Sc17.

Figure 7.3. Dusty black coloration in the agar underneath the interacting zone of complementation reaction of Pf67 and Sc17.

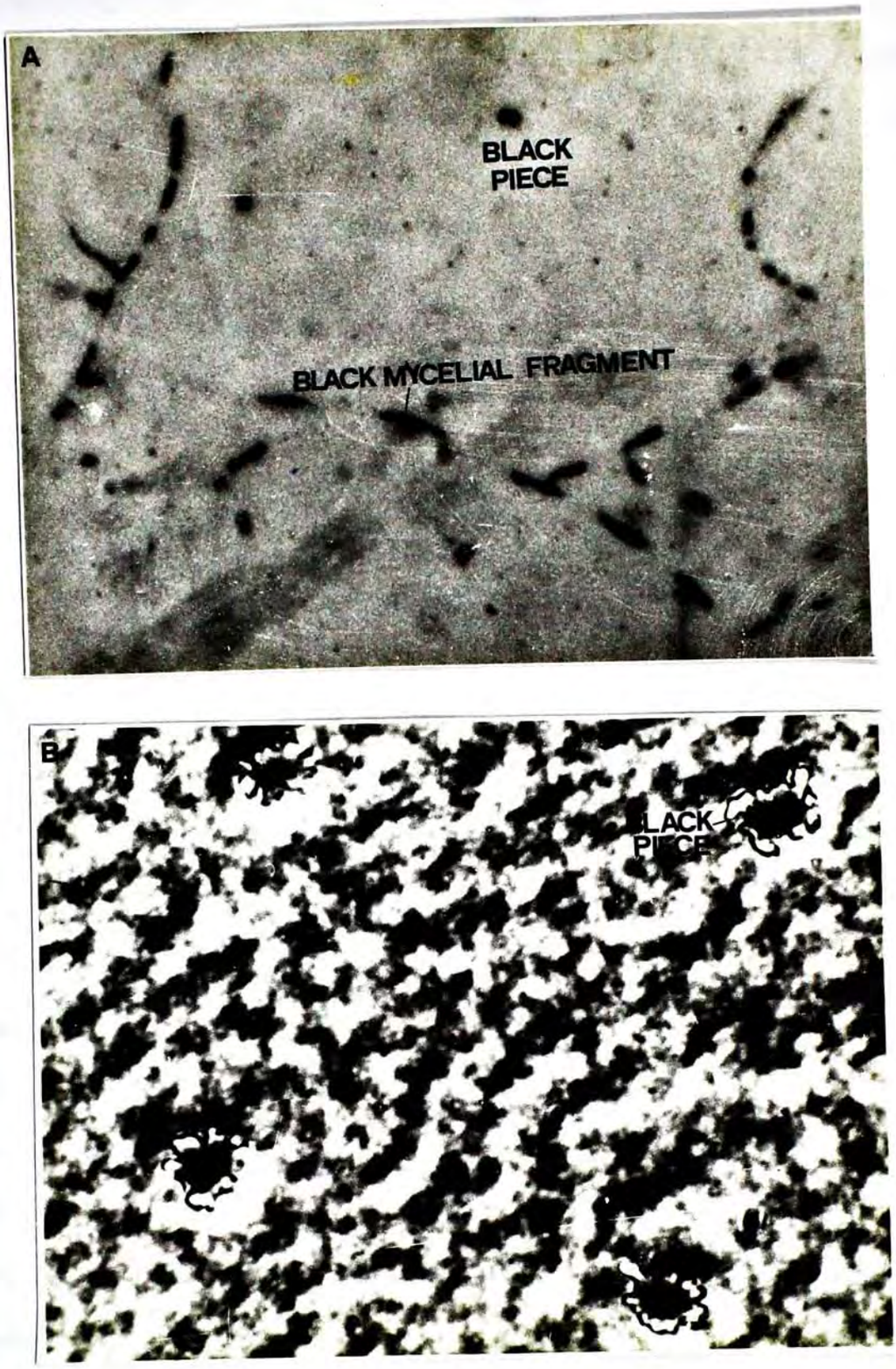


Figure 7.4. Microscopic observations of the coloration in agar underneath the interacting zone of complementation test between Pf67 and Sc17. (a) 200 × micrograph showed the black particulate substances inside the agar. The darkened mycelia might be a possible source of the black particulate substances. (b) 800 × micrograph showed the enlarged view of the black particulate substances.

For the complementation analysis of the spore germinants of PS1, the results of the test for the spore germinants of the three PS1 fruit bodies was shown in table 7.8.

Table 7.8. The number of mating reactions belongs to different types of mating reaction.

Type of mating reaction	+	F	B	-	$*\Sigma \frac{(O-E)^2}{E}$
First fruit body	47	41	43	40	0.68
Second fruit body	42	44	61	36	7.7
Third fruit body	48	54	43	35	4.30

* : The chi-square value ($\times^2$).

Chi-square test was then carried out for the above result. The null hypothesis was set to be H_0 = The ratio of + : F : B : - fits the 1 : 1 : 1 : 1 ratio. H_1 = The ratio of + : F : B : - has a different ration other than the 1 : 1 : 1 : 1 ratio. With the degree of freedom equal to 3, the critical value $\times^2_{0.05}$ for a 0.05 level of significance was found to be equal to 7.81. Therefore, all of the $\times^2$ for the three fruit bodies were smaller than the $\times^2_{0.05}$ value. Hence, we do not reject the null hypothesis that the ratio of + : F : B : - fits the 1 : 1 : 1 : 1 ratio.

7.3.3. Monokaryotic protoplast regenerants of dikaryotic fusion product PS1

From two independent batches of experiment, protoplast isolation of PS1 under the condition described in section 7.2.3. resulting in a solution with mean

protoplast concentration of 5 × 10^5 protoplasts per milliliter. The mean regeneration frequency of protoplasts was found to be 0.53 %. In 100 colonies inspected, forty-three colonies were subcultured and only thirty-one out of them were finally identified as monokaryons. Therefore, 31 % of the regenerants were found to be monokaryotic (without true clamp connection) and 69 % of the regenerated protoplasts gave rise to a majority of dikaryotic mycelia (with true clamp connection). For characterization of the mating types of the monokaryotic regenerants, the M4 monokaryotic regenerants was chosen as the M× tester and the M9 strain were found to be the compatible strain to M4 monokaryotic regenerants. Therefore, M9 strain became the M×' tester. Out of these thirty-one monokaryotic regenerants, twenty-one of them showed to have positive mating reaction (presence of clamp connection) with M4 monokaryotic regenerants but have negative reaction with M9 strain. All the other eight monokaryotic regenerants showed to have compatible mating type with the M9 monokaryotic regenerants but negative reaction with M4. The former group of monokaryotic regenerants were entitled to be "M9 type" monokaryotic regenerants as they have the same mating type with M9 strain. The latter group of monokaryotic regenerants were then be the "M4 type" monokaryotic regenerants. For the mating test using Sc17 and Pf67 as tester strains, all M4 type monokaryotic regenerants found to be non-compatible with both Sc17 and Pf67. However, all M9 type monokaryotic regenerants showed to have positive reaction only with Sc17 but none with Pf67. In addition, all the nine M4 type monokaryotic regenerants showed to form their mycelial colonies with a pale-pink color and as a thin mycelial mat with non-dense aerial hyphae. Such a pale-pink colonial coloration and the non-dense aerial hyphae morphology of the colony were also some characters of Sc17.

7.3.4. Studies on extraction of undigested genomic DNA

The results on the qualitative study of undigested genomic DNA showed fluorescence intensity of the DNA sample of the first two lanes were higher than that of the third and the forth lanes (figure 7.5.). It indicated that the amount of DNA isolation from 0.3 mg wet weight of Pf67 by Lee and Taylor's (1990) method was more than that extracted by Yoon *et al.*'s (1991) method. On the aspect of RNA removal by RNAase, most of RNA in the genomic DNA sample was shown to be removed after digestion by RNAase (figure 7.6.). By the method described by Yoon et al., genomic DNA for both fusion parents and fusion products was extracted. It was found that the DNA sample of most strains showed to give clear bands without extensive smearing in low molecular weight region (figure 7.7.). Most of the bands were found to have their molecular weight about 23.1 kilobases. Only very faint bands were observed in the forth and the fifth lanes which were corresponding to the DNA samples of PS1. Therefore, the extraction method was applicable to most strains except PS1.

Preliminary AP-PCR amplification test was carried out in order to determined whether the two DNA methods able to produce DNA which was suitable for generating genomic fingerprints for comparison. It was found that the fusion parents DNA extracted by both methods able to generate their corresponding genomic fingerprints (figure 7.8., figure 7.9.). However, for the fusion parent Sc17, the DNA profile of its fingerprint for the Lee and Taylor (1990) method were crowded in the low molecular weight range region (figure 7.8.). However, genomic fingerprints of Pf67 from the same DNA extraction method had the DNA profile spanned through the large molecular size range. This later situation could also be observed for the DNA profile of both Pf67 and Sc17 from the Yoon *et al.*'s method (figure 7.9.).

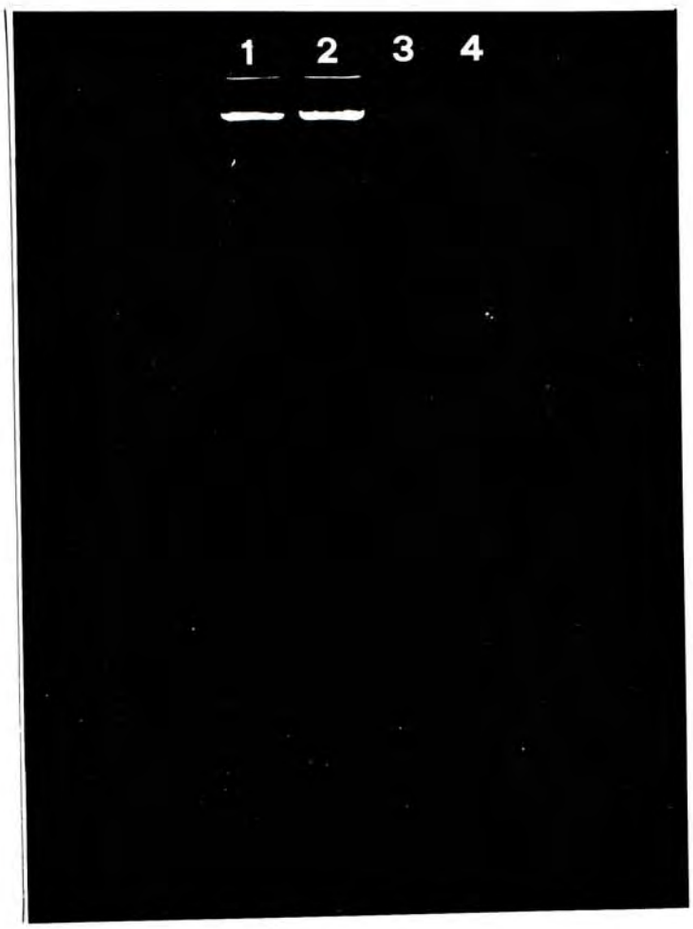


Figure 7.5. Agarose gel electrophoresis of undigested genomic DNA from *Pleurotus florida* Pf67; stained with ethidium bromide and viewed by UV light. DNA samples were loaded on a 0.8 % agarose gel containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 30 minutes at 7 volts/cm. Lane 1-2: DNA isolated by Lee and Taylor's (1990) method using chloroform : TE-saturated phenol (1:1;v:v) solution for removal of impurities. Lane 3-4: DNA isolated by Yoon *et al.*'s (1991) method employing CsCl to purify DNA from fungal tissue.

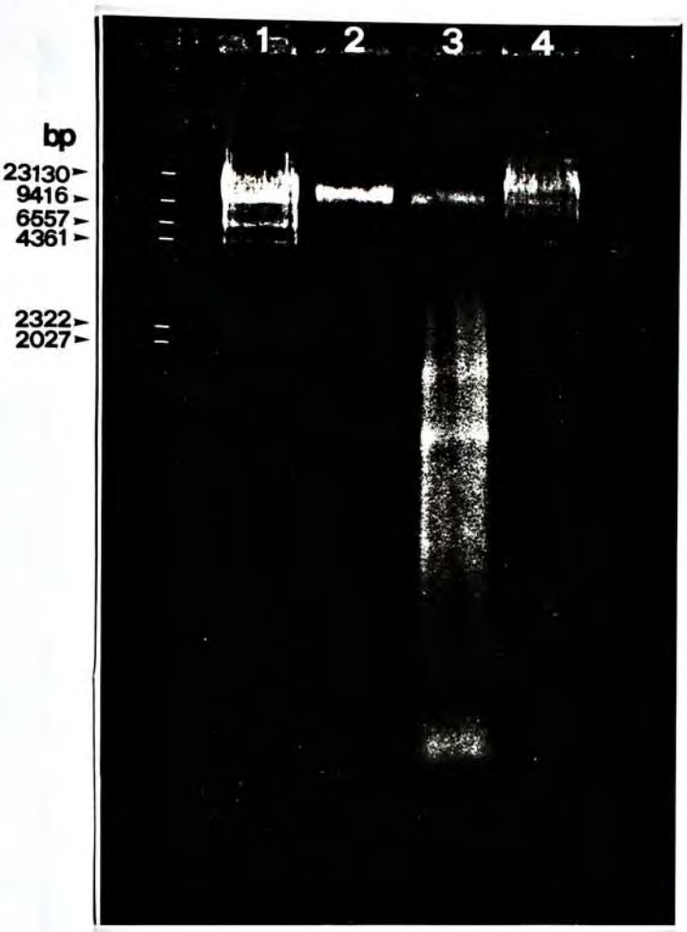


Figure 7.6. Agarose-gel electrophoresis of undigested genomic DNA from *Pleurotus florida* Pf67 isolated by Yoon *et al.*'s (1991) method. DNA was stained with ethidium bromide and viewed by UV light. DNA samples were loaded on a 0.8 % agarose gel containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 1 hour at 7 volts/cm. Lane 1: λ DNA digested with Hind III as standards; Lane 2: DNA isolated from Pf67 with removal of RNA by RNAase; Lane 3: DNA isolated from Pf67 without RNA digestion by RNAase; Lane 4: λ DNA digested with Hind III as standards.

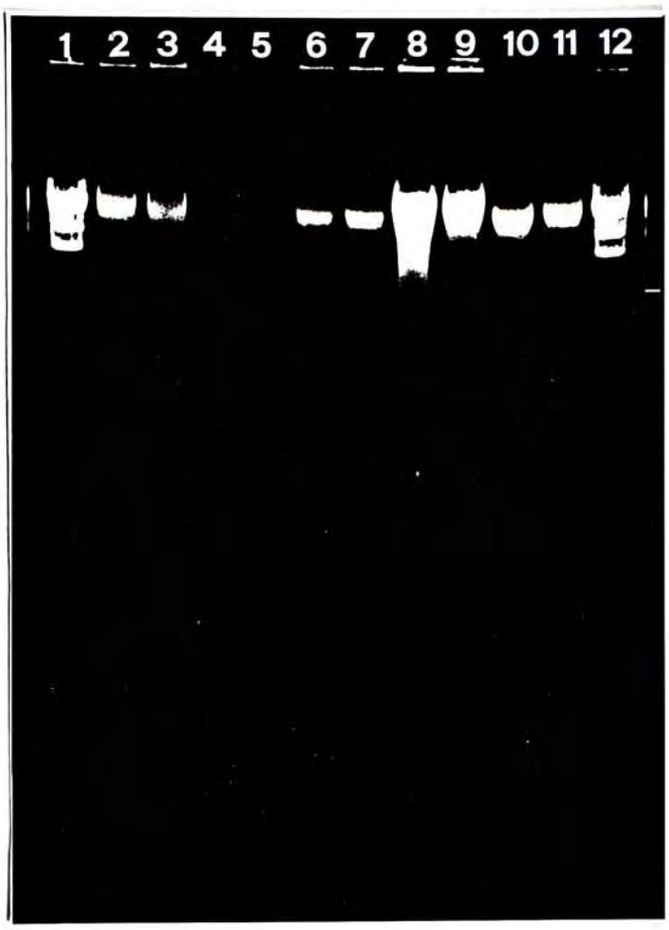


Figure 7.7. Agarose-gel electrophoresis of undigested genomic DNA, which was isolated by Yoon *et al.*'s (1991) method, from various strains. DNA was stained with ethidium bromide and viewed by UV light. DNA samples were loaded on a 0.8 % agarose gel containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 1 hour at 7 volts/cm. Lane 1: λ DNA digest with Hind III as standard; Lane 2-3: *Pleurotus florida* Pf67; Lane 4-5: fusion product PS1; Lane 6-7: fusion product PS2; Lane 8-9: fusion product PS3; Lane 10-11: *Schizophyllum commune* Sc17; Lane 12: λ DNA digest with Hind III as standard.

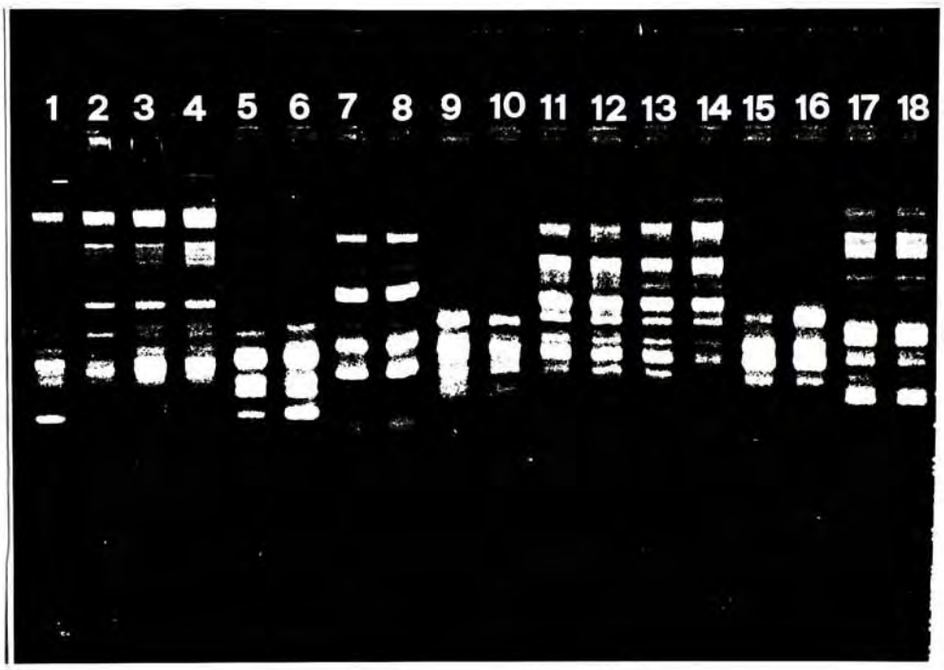


Figure 7.8. Genomic fingerprints of *Pleurotus florida* Pf67 and *Schizophyllum commune* Sc17, AP-PCR patterns were different for the same strain when the primer used for the DNA amplification was different. The genomic DNA of both Pf67 and Sc17 were extracted by Lee and Taylor's (1990) method. The resulting amplified materials were resolved by electrophoresis through 3 % agarose containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 2 hour at 7 volts/cm. DNA bands were visualized by staining with ethidium bromide and viewed under UV light. Lane 1-4: Pf67, M13 forward sequencing primer (-47); Lane 5-6: Sc17, M13 forward sequencing primer (-47); Lane 7-8: Pf67, M13 reverse sequencing primer (-48); Lane 9-10: Sc17, M13 reverse sequencing primer (-48); Lane 11-14: Pf67, Gal K; Lane 15-16: Sc17, Gal K; Lane 17-18: Pf67, EcoRI-Ext.

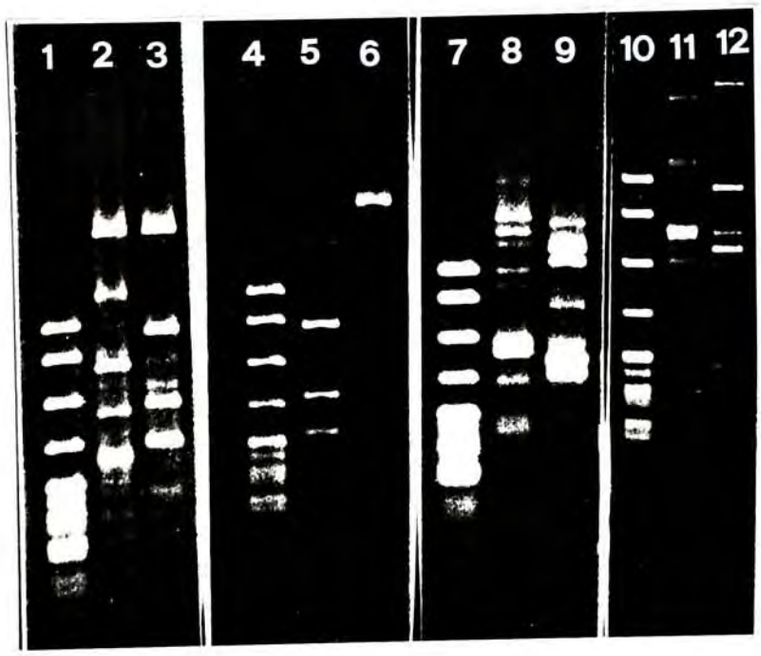


Figure 7.9. Genomic fingerprints of *Pleurotus florida* Pf67 and *Schizophyllum commune* Sc17. AP-PCR patterns are different for the same strain when the primer used for DNA amplification was different. The genomic DNA of both Pf67 and Sc17 were extracted by Yoon *et al.*'s (1991) method. The resulting amplified materials were resolved by electrophoresis through 3 % agarose containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 2 hours at 7 volts/cm. DNA bands were visualized by staining with ethidium bromide and viewed under UV light. Lane 1, 4, 7, 10: pBR322 DNA digested by MspI as standard; Lane 2: Pf67, M13 forward sequencing primer (-47); Lane 3: Sc17, M13 forward sequencing primer (-47); Lane 5: Pf67, M13 reverse sequencing primer (-48); Lane 6: Sc17, M13 reverse sequencing primer (-48); Lane 8: Pf67, EcoRI-Ext; Lane 9: Sc17, EcoRI-Ext; Lane 11: Pf67, Gal K; Lane 12: Sc17, Gal K.

7.3.5. Genomic fingerprinting by AP-PCR

Genomic fingerprints of all fungal strains were successfully generated except those of PS1. Only faint DNA bands can be observed in all four genomic fingerprints of PS1. The DNA profiles of the genomic fingerprints of both fusion parents and fusion products as well as the M4 and M9 type regenerants were shown in figure 7.10. to figure 7.14..

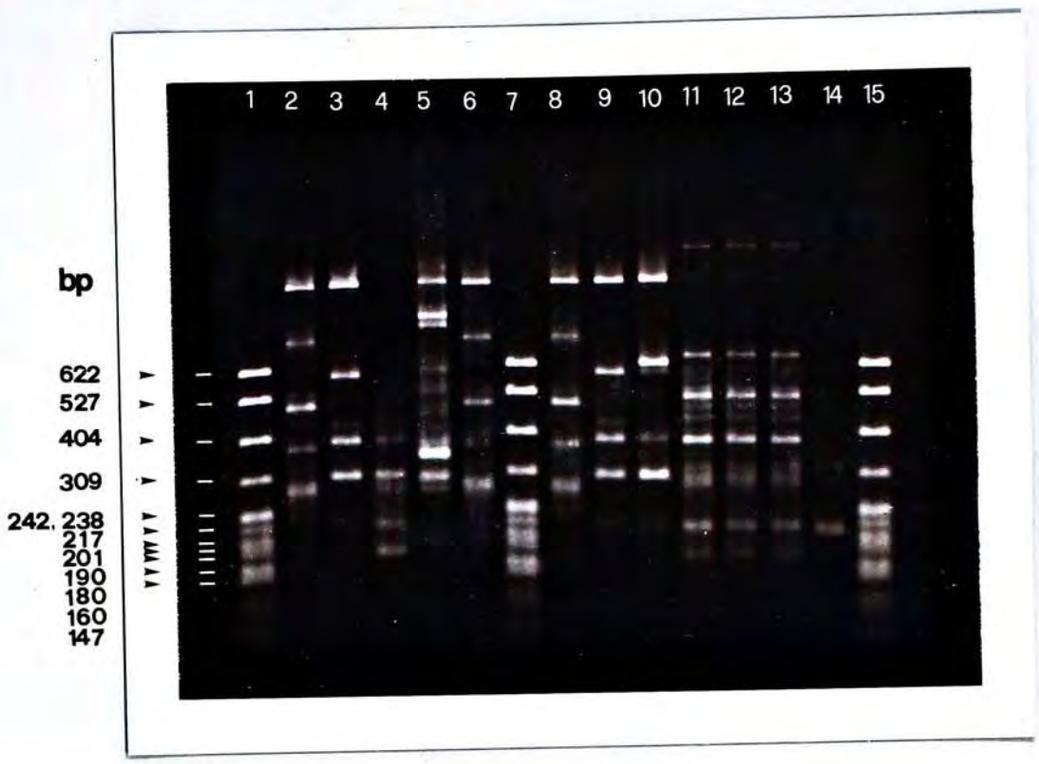


Figure 7.10. Genomic fingerprints generated by AP-PCR using M13 forward sequencing primer (-47). The genomic DNA of all strains were extracted by Yoon *et al.*'s (1991) method. The resulting amplified materials were resolved by electrophoresis through 3 % agarose containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 2 hours at 7 volts/cm. DNA bands were visualized by staining with ethidium bromide and viewed under UV light. Lane 1, 7, 15: pBR322 DNA digested by MspI as standard; Lane 2,8: Pf67; Lane 3,9: Sc17; Lane 4: PS1; Lane 5: PS2; Lane 6: PS3; Lane 10: M4-1; Lane 11: M9-1; Lane 12: M9-2; Lane 13: M9-3; Lane 14: Control (AP-PCR without template DNA).

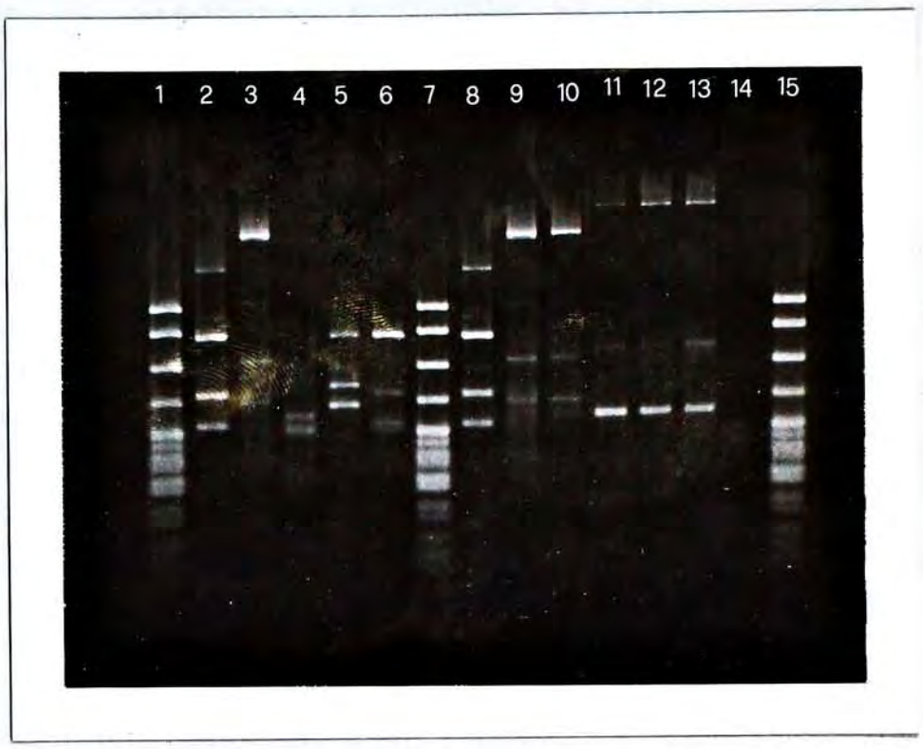


Figure 7.11. Genomic fingerprints generated by AP-PCR using M13 reverse sequencing primer (-48). The genomic DNA of all strains were extracted by Yoon *et al.*'s (1991) method. The resulting amplified materials were resolved by electrophoresis through 3 % agarose containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 2 hours at 7 volts/cm. DNA bands were visualized by staining with ethidium bromide and viewed under UV light. Lane 1, 7, 15: pBR322 DNA digested by MspI as standard; Lane 2,8: Pf67; Lane 3,9: Sc17; Lane 4: PS1; Lane 5: PS2; Lane 6: PS3; Lane 10: M4-1; Lane 11: M9-1; Lane 12: M9-2; Lane 13: M9-3; Lane 14: Control (AP-PCR without template DNA).

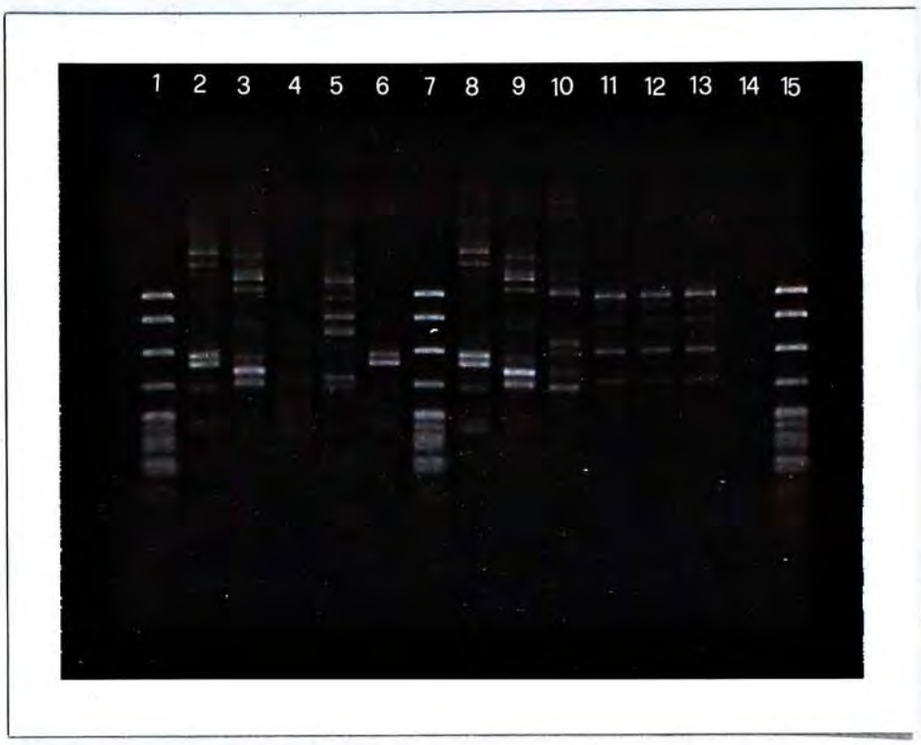


Figure 7.12. Genomic fingerprints generated by AP-PCR using EcoRI-Ext primer. The genomic DNA of all strains were extracted by Yoon *et al.*'s (1991) method. The resulting amplified materials were resolved by electrophoresis through 3 % agarose containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 2 hours at 7 volts/cm. DNA bands were visualized by staining with ethidium bromide and viewed under UV light. Lane 1, 7, 15: pBR322 DNA digested by MspI as standard; Lane 2,8: Pf67; Lane 3,9: Sc17; Lane 4: PS1; Lane 5: PS2; Lane 6: PS3; Lane 10: M4-1; Lane 11: M9-1; Lane 12: M9-2; Lane 13: M9-3; Lane 14: Control (AP-PCR without template DNA).

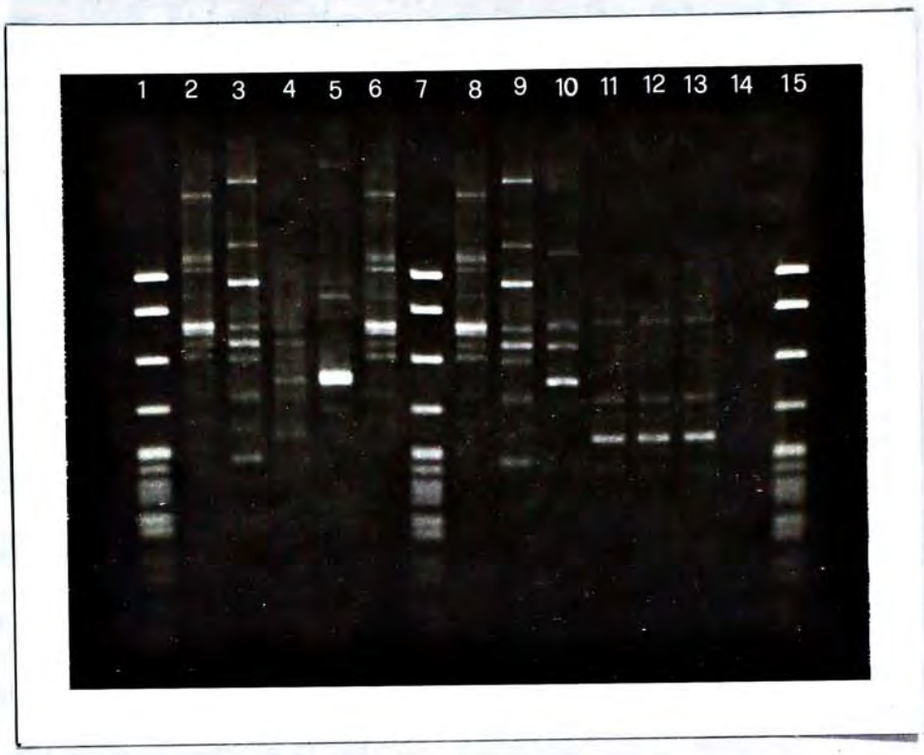


Figure 7.13. Genomic fingerprints generated by AP-PCR using Gal K primer. The genomic DNA of all strains were extracted by Yoon *et al.*'s (1991) method. The resulting amplified materials were resolved by electrophoresis through 3 % agarose containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 2 hours at 7 volts/cm. DNA bands were visualized by staining with ethidium bromide and viewed under UV light. Lane 1, 7, 15: pBR322 DNA digested by MspI as standard; Lane 2,8: Pf67; Lane 3,9: Sc17; Lane 4: PS1; Lane 5: PS2; Lane 6: PS3; Lane 10: M4-1; Lane 11: M9-1; Lane 12: M9-2; Lane 13: M9-3; Lane 14: Control (AP-PCR without template DNA).

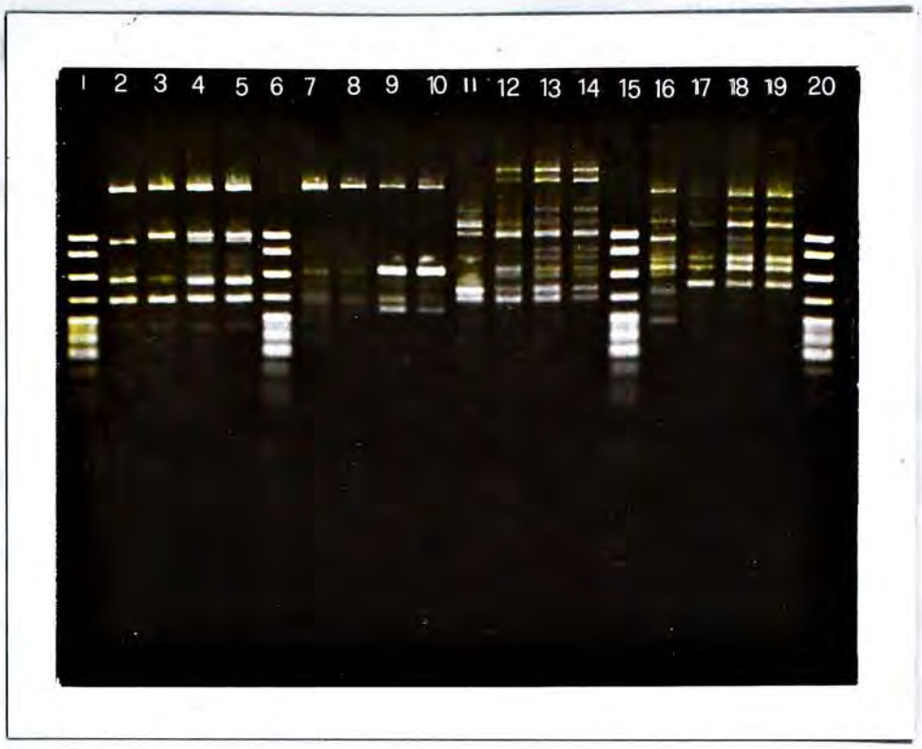


Figure 7.14. Genomic fingerprints of Schizophyllum commune Sc17 and M4 type regenerants generated by AP-PCR using four types of primer. The genomic DNA of all strains were extracted by Yoon *et al.*'s (1991) method. The resulting amplified materials were resolved by electrophoresis through 3 % agarose containing 0.045 mM Tris-borate and 0.001 M EDTA and run for 2 hours at 7 volts/cm. DNA bands were visualized by staining with ethidium bromide and viewed under UV light. Lane 1, 6, 15, 20: pBR322 DNA digested by MspI as standard; Lane 2: Sc17, M13 forward sequencing primer (-47); Lane 3: M4-1, M13 forward sequencing primer (-47); Lane 4: M4-2, M13 forward sequencing primer (-47); Lane 5: M4-3, M13 forward sequencing primer (-47); Lane 7: Sc17, M13 reverse sequencing primer (-48); Lane 8: M4-1, M13 reverse sequencing primer (-48); Lane 9: M4-2, M13 reverse sequencing primer (-48); Lane 10: M4-3, M13 reverse sequencing primer (-48); Lane 11: Sc17, EcoRI-Ext; Lane 12: M4-1, EcoRI-Ext; Lane 13: M4-2, EcoRI-Ext; Lane 14: M4-3, EcoRI-Ext; Lane 16: Sc17, Gal K; Lane 17: M4-1, Gal K; Lane 18: M4-2, Gal K; Lane 19: M4-3, Gal K.

7.4. Discussions

7.4.1. Genomic DNA extraction

The success of molecular analysis using enzymatic amplification of DNA sequences was critically dependent on the quality of the extracted DNA. It was because contaminants, for instance, polysaccharides from cell wall, might interfere with the activity of DNA polymerase (Murray and Thompson, 1980). Therefore, effective DNA extraction method was important for obtaining purified DNA for enzymatic DNA amplification reaction.

A number of methods have been published for isolation of fungal DNA. Two methods were carried out and qualitative comparison was made. Both Lee and Taylor's (1990) method and Yoon *et al.*'s (1991) method showed to be able to isolate genomic DNA in high quality (figure 7.5.). The amount of DNA isolated from Lee and Taylor's method was relatively higher. However, on the aspects of DNA degradation, no extensive degradation was found in the resulting DNA isolated from both methods. Considering the DNA profiles of the AP-PCR fingerprints of Sc17, fingerprints generated from all four types of primer showed to span only within a narrow range of molecular weight (figure 7.8.). For the purpose of fingerprinting the genome of a fungal strain, the amplification products should be better to involve both high and low molecular weights DNA sequences. Moreover, the major bands of the profile should be separated clearly. Although the genomic fingerprints of Pf67 in figure 7.8. showed to be acceptable based on the criteria mention above, Sc17 genomic fingerprints were not suitable for the present purpose.

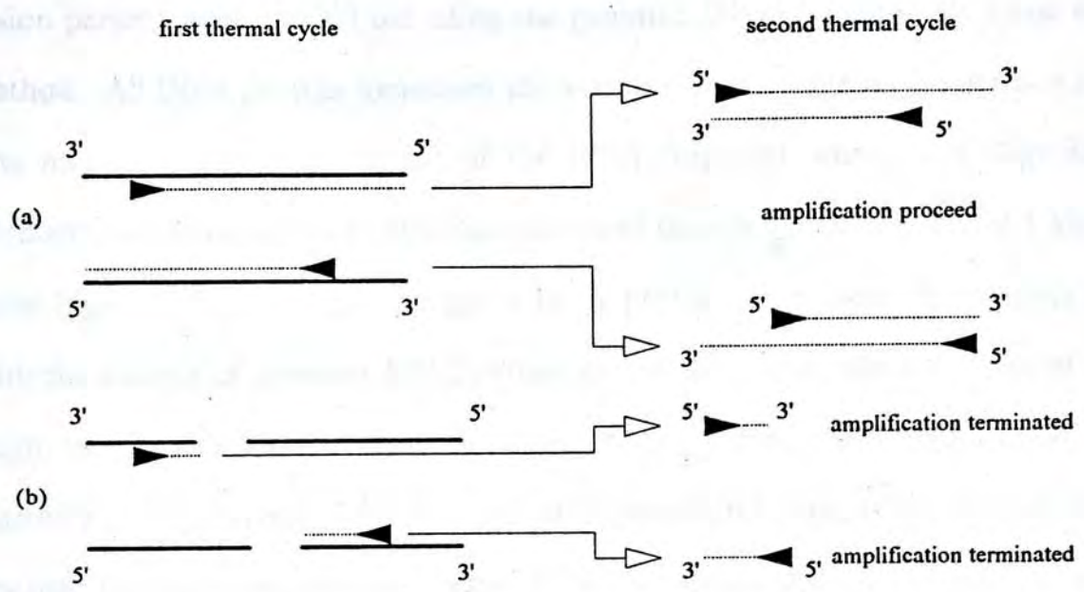


Figure 7.15. DNA amplification with genomic DNA as template. (a) Complete double strands genomic DNA involved two priming sites. The newly synthesized DNA strand act as template for the formation of the complementary strand in the second thermal cycle. (b) The double strands genomic DNA was broken by mechanical damage. The template formed in the first thermal cycle have no priming site for primer hybridization in the second thermal cycle. Therefore, DNA amplification ceases.

Caesium chloride technique mentioned by Yoon *et al.* was a rapid and simple method and also claimed to be effective. The improvement in shortening the lengthy procedure of DNA extraction should theoretically minimize the mechanical degradation of genomic DNA. Actually, when genomic fingerprints of the two fusion parents were carried out using the genomic DNA extracted by Yoon et al.'s method. All DNA profiles generated showed to cover a large molecular size range. The minimum molecular weight of the DNA fragment among the amplification products was approximately 200 base pairs and the maximum was about 1 kilobase pairs (figure 7.9.). Such a change in DNA profile of genomic fingerprints along with the change of genomic DNA extraction method, especially for those of Sc17, might be due to a lot of factors. The lengthy procedure in DNA extraction which caused a certain extends of mechanical damages might be one of the factors. It was because the clustering of DNA bands in Sc17 genomic fingerprints around the low molecular weight region reflected that no high molecular weight product was generated in the first two thermal cycles. Therefore, the mechanical breakage might occur somewhere along the genomic DNA sequences which separate the two priming sites (figure 7.10.), the formation of the corresponding amplification product became impossible.

Other factors such as the impurity, for instance, phenol as well as proteins, might also contribute for the phenomenon. Besides the improvement of DNA profiles in genomic fingerprints by using the Yoon *et al.*'s DNA extraction method, it should be noted that the whole DNA profiles of both Pf67 and Sc17 were changed when the DNA extraction method was changed. Therefore, the quality of DNA sample might also be a factor in determining the extended of the relaxed stringency condition in the first two cycles of the AP-PCR process. Therefore, all experiment with an objective for comparing AP-PCR fingerprints, the DNA

extraction procedures should better be standardized for all fungal stains. Considering all aspects mentioned above, Yoon *et al*'s method was employed in this study.

On the aspect of RNA removal, the present condition was found to be effective to remove most of the RNA from the DNA sample (figure 7.6.). The general method for RNA degradation was adding RNAase after the whole DNA extraction procedure was completed. However, in the present protocol, inserting the RNA degradation steps before CsCl treatment made it not necessary to repeat the DNA purification step after the DNA was resuspended in TE buffer after the whole extraction procedure. Such a design had an advantage of shortening the lengthy procedure for DNA preparation and hence lessened the chance of DNA degradation during processing.

The results of DNA extraction showed in figure 7.7. indicated that the Yoon *et al.*'s method was unable to extract PS1 genomic DNA. Actually, the dikaryotic strain PS1 produced a large amount of carbohydrate which coprecipitated with DNA in isopropanol. Redissolving the carbohydrate-DNA precipitate in TE resulted in a gelatinous solution. Such a phenomenon did not occur in the DNA extraction of the monokaryotic *Schizophyllum commune* Sc17 or *Pleurotus florida* Pf67. Actually, levels of intracellular water-soluble polysaccharides, such as glycogen, in both monokaryotic and dikaryotic strains of *Schizophyllum commune* have been reported to be similar (Mattila and Raudaskoski, 1992). Therefore, the polysaccharides was likely to be mostly presence in the extracellular pool. To solve the problem, alternation in DNA extraction procedure specific for PS1 was not feasible. Therefore, haploidization was carried out as an attempt to recover the individual nuclear types of the dikaryotic PS1. Actually, it was necessary for

interpreting the genomic fingerprints of the two nuclear types of PS1 separately! It was because the genomic fingerprint of DNA sample from "mixed" genome would make the comparison between PS1 with other strain more complicated. The level of polysaccharide, which coprecipitated with DNA in isopropanol, in both M4 type and M9 type regenerants was found to be much lesser than their dikaryotic parent PS1. No observable coprecipitation of polysaccharides during DNA precipitation in isopropanol can be observed for both M4 and M9 type regenerants.

7.4.2. Recovery of the individual nuclear type of dikaryotic fusion product PS1

The regeneration frequency of PS1 protoplast was found to be similar to that of Pf67 and Sc17, which was about 0.5 %. However, regeneration of PS1 protoplasts gave rise to a majority of dikaryotic mycelia (69 %). Therefore, the result indicated that the two nuclei in PS1 mycelial cell were located in close proximity and hence the two nuclei often captured in a single protoplast. However, separation of the two nuclei through protoplasting was still possible. The formation of monokaryotic mycelia from the 31 % of protoplasts indicated that part of the protoplasts only captured a single nucleus inside. Wessels *et al.* (1976) have been reported a comparable result of 20 to 40 % monokaryon among the protoplast regenerants of the dikaryon of *Schizophyllum commune*. Unfortunately, they reported no information on the number of the two individual nuclear types among the monokaryotic regenerants. In my research, the M4 type to M9 type monokaryotic regenerants' ratio was about 1 to 3. Such result was found to be deviated from the expected 1 to 1 ratio. It might be due to the fact that the mycelial growth rate of M9 type regenerants was faster than that of the M4 type regenerants. Therefore, the chance to fish out the M9 type regenerants became higher.

However, based on the results obtained so far, we still could not eliminate the possibility that this ratio really reflected the fact that the regeneration abilities of M4 type regenerants were relatively lower.

Considering the mating type compatibility of the two nuclear type regenerants with the fusion parents, the result (table 7.7.) showed that M4 type regenerants had their mating type gene similar to that of Sc17 but those of M9 type were different from that of Sc17. The formation of the true clamp connections in the mating reactions between the M9 type regenerants and Sc17 implied that the A- and B- incompatibility factors of the former and the latter strain were in different allelic forms. On the other hand, the inability of both M4 and M9 type regenerants to form clamp connections with Pf67 indicated that the A- and B- incompatibility factors of all three strains might be the same! However, the result of mating reaction of M4 and M9 type with Sc17 already showed that their incompatibility factors were different ($A \neq B \neq$). Therefore, the incompatibility factors of the monokaryotic regenerants of M4 and M9 type regenerants might not be in allelic form of that of Pf67. Actually, the interaction morphologies of the M4 and M9 type regenerants to Pf67 were found to be similar to that between Sc17 and Pf67. All them showed to have a "climbing" morphology (figure 7.2) along the interaction zone as well as the formation of black dusty substances inside the agar underneath the interaction zone (results from section 7.3.2.). From the results of both mating tests and interaction morphologies in mating reactions, both M4 and M9 type regenerants had their incompatibility factors exist in allelic forms of those in *Schizophyllum commune*. However, no evidence indicated for the presence of Pf67's incompatibility factors in both M4 and M9 type regenerants.

Nevertheless, through the process of protoplast release, the two nuclear types in the dikaryon were separated. The two corresponding monokaryotic regenerants' groups, M4 type and M9 type, were obtained.

7.4.3. Genomic changes in fusion products

The genomic changes in fusion products relative to the two fusion parents were revealed by comparing their corresponding AP-PCR fingerprintings. To fingerprint the genomic DNAs from the eleven strains, we used four different arbitrary primers. They are M13 forward sequencing primer (-47) (M13 seq), M13 reverse sequencing primer (-48) (M13 RS), EcoRI-Ext primer and Gal K primer. In order to confirm DNA bands were generated from amplification of genomic DNA and not the primer artifact, genomic DNA was omitted from control reactions for each primer. No amplification products were seen for any primer except for primer M13 forward sequencing primer (-47) (figure 7.10., lane 14); this artifact was generally known to be the concatemer of the primer itself. Actually, the molecular weight of these concatemer was very low. Therefore, these artifact can easily be identified in the DNA profile. The results of figure 7.10. to figure 7.14. showed that single primers of arbitrary sequence can be used to amplify genomic DNA segments from all fusion parents, fusion products as well as the monokaryotic regenerants of PS1, except the dikaryotic fusion product PS1. The poor efficiency of the presence DNA extraction method on this strain and the existing of large amount of carbohydrate in the corresponding DNA sample has already been discussed in section 7.4.1.. Therefore, the inability of generating AP-PCR fingerprinting by all four primers for the strain was expected.

To determine whether AP-PCR analysis was sensitive enough to reveal differences between the two fusion parents, preliminary AP-PCR was carried out with the use of DNA samples of the two parents collected from different DNA extraction method, the results demonstrated that no matter which type DNA extraction method was employed, the DNA profiles of the AP-PCR fingerprints of the two parents were mostly different from each other (figure 7.8. and figure 7.9.). Many of these different were present among the major fragments although closer examination revealed a few number of similar bands in the less prominent minor or background fragments.

For confirmation of the presence of change(s) in genomic content in fusion products relative to the fusion parents, the DNA profiles of the AP-PCR fingerprints of each fusion product were compared to that of the two fusion parents. For PS2, all four fingerprints demonstrated a whole range of different amplification products relative to the two fusion parents except the highest molecular weight band of M13 seq fingerprint which was common for both parents, the 515 base pairs' band of M13 RS fingerprint which was similar to that of Pf67 fingerprint and the 300 base pairs' band of EcoRI-Ext fingerprint which was similar to that of Pf67 fingerprint. Therefore, extensive genetical rearrangements might occur in the nucleus of PS2 after the protoplast fusion process. For the formation the such genetical rearrangements, nuclear fusion might be occurred before the process of such genetical changes. Therefore, the partly or whole genetical materials of the two fusion parents might present inside the same nucleus and interaction became possible.

Considering the AP-PCR fingerprints of the fusion product PS3, both the M13 seq and the Gal K fingerprints patterns of PS3 and Pf67 were demonstrated to

be mostly the same. For the M13 RS and EcoRI-Ext fingerprint of PS3, they only demonstrated to be part of that of Pf67's. The similarity of the former two fingerprints with that of Pf67's suggested that the present of Pf67 genomic DNA in the genome of PS3 was highly probable. However, the latter two fingerprints implicated that there might be some sequence changes at the priming sites, which were responsible for the amplification of the missing bands (all bands greater than 527 base pairs of EcoRI-Ext fingerprint and the highest molecular weight band of the M13 RS fingerprint of Pf67). On the other hand, no new band or any band similar to that of Sc17 was found in the amplified DNA fragments profiles of PS3. Therefore, no evidence was found for supporting the presence of the genomic materials of Sc17 in genome of PS3.

For the two groups of monokaryotic regenerants of the PS1, the AP-PCR fingerprints from all four primers of the three strains in ea ch group demonstrated to be mostly similar. Therefore the genomic contents of the strains in each group were likely to be similar too. For the M4 type regenerants, the corresponding fingerprints of M13 seq and M13 RS demonstrated to have the whole sets of the corresponding fingerprint bands of Sc17. In addition to the original DNA fragment bands of Sc17, M4 type regenerants showed to have an extra band in the M13 seq fingerprint (622 base pairs). This extra band was a new band which did not occur in both parents. Therefore, there might be two new primers annealing sites occurred in M4 type regenerants relative to Sc17. The EcoRI-Ext fingerprint of both M4 type regenerants and Sc17 possessed 8 amplified fragments, however, only three of them had the same molecular weight, all other five bands were different for the two strains. None of these five bands showed to have similar molecular weight with that of Pf67's. The Gal K fingerprint of Sc17 had 8 major bands and two minor bands. However, the Gal K fingerprint of M4 type regenerants showed to have only 4

major bands and 2 minor bands. Only one major band was found to be common for both parents as well as M4 type regenerants. All other different bands were new DNA fragments. Therefore, some fingerprints of the M4 type regenerants were highly similar to that of Sc17 and some fingerprints showed to have partly homology between them. Therefore, the existing of the Sc17 genomic material in M4 type regenerants of PS1 was highly probable. The new bands implied the existing of extra primer annealing site. This change might be due to the change in original DNA sequence of the genomic materials of Sc17 genomic materials in M4 type regenerants. On the other hand, we could not rule out the possibility that new genetic materials, such as the genomic materials of Pf67, enters the nucleus of M4 type regenerants by some means and resulting in formation of the new amplified DNA fragments! Similar to the situation of PS3, none of the above results demonstrated the coexisting of the genetic materials of the two fusion parents in M4 type regenerants of PS1. For the fingerprints of the M9 type regenerants of PS1, the results were similar to those of PS3. All four fingerprint patterns demonstrated a whole range of different amplification products. Exceptions could only be found in the M13 seq and Gal K fingerprints. Only one band in each of the two fingerprints showed to be in common with that of Sc17. Therefore, the results of the M9 type regenerants also indicated that there might be some kind of changes in genomic materials in the strains after the protoplast fusion process.

In the above comparisons of the AP-PCR fingerprints of the eleven strains, it was found that loss of the genomic materials of either of the two fusion parents in the fusion product, such as the Pf67 genomic materials in PS3 and Sc17 genetic materials in M4 type regenerants of PS1, were highly probable. However, the fact that some kind of genetical changes occurred in the genome of the fusion products could not be denied.

7.4.4. Progeny analysis and determination of auxotrophic as well as drug resistance markers

The results of progeny analysis were summarized in figure 7.16.. From these results, the segregation patterns of the auxotrophic and drug resistance markers from the fusion parents to the progenies of the dikaryotic fusion product PS1 were studied. Considering the auxotrophic markers, the phenotype of the dikaryotic fusion product PS1 was found to be adenine requiring. The fruit body of PS1 was morphologically similar to those of *Schizophyllum commune*. Therefore, it was assumed that the genetic materials of Sc17 were predominantly present in the genomes of the two nuclei of PS1.

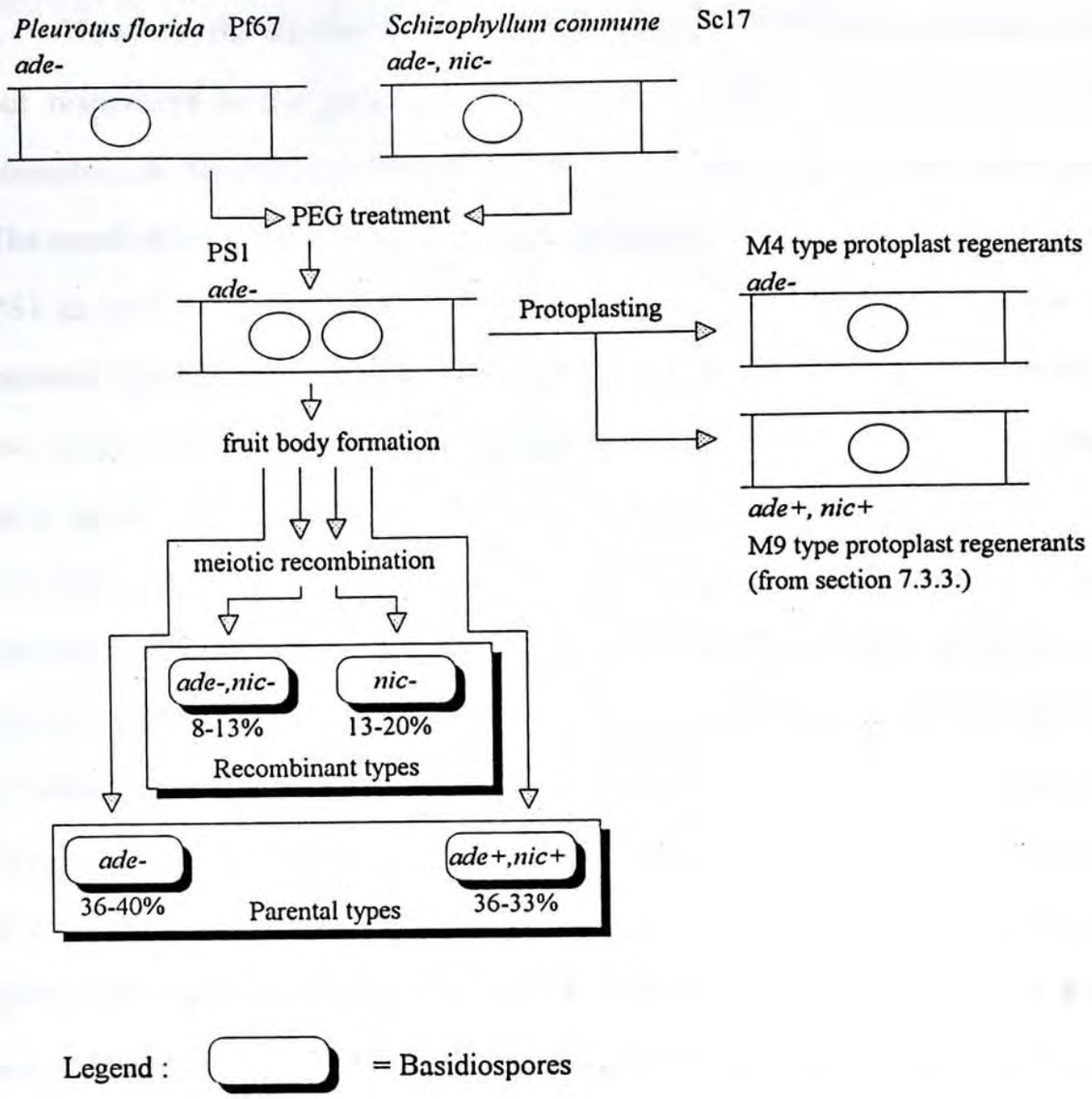


Figure 7.16. The outline of results from the experiment mention in section 7.2.1.. The phenotypes of M4 type and M9 type regenerants supported the parental types phenotypes found in the monosporus isolates from fruit body of PS1. Although nicotine requiring marker was "disappeared" in PS1, this marker reappeared in the progenies of PS1 through meiotic recombination.

However, the nicotine requiring (*nic*$^-$) marker of Sc17 was not found in PS1 but reappeared in the progenies of PS1 (figure 7.16.). To account for such phenomenon, the phenotypes of the two nuclear types of the PS1 was investigated. The result of both M4 type and M9 type regenerants in protoplasting experiment of PS1 as well as the study of monosporous isolates of PS1 showed that the two parental types of the progenies were (1) *ade*$^-$ and (2) *ade*$^+$, *nic*$^+$. The change of *nic*$^-$ in Sc17 to *nic*$^+$ phenotye in PS1 might be due to a lot of reasons. There might be a genetic recombination of the nicotinic acid gene. If it had happened, the recombination might not occur between the nicotine genes of Pf67 and Sc17 because they were existed in non-allelic forms. Another alternative possibility was the presence of both *nic*$^-$ gene of Sc17 and *nic*$^+$ gene of Pf67 in the same genome of either or both nucleus type of PS1. Therefore, when the both nicotine genes expressed in the same nucleus, the resulting phenotype caused by the *nic*$^+$ gene of Pf67 might mask the effect of the *nic*$^-$ gene of Sc17 and showed a nicotin requiring phenotype. After meiotic recombination in basidium of fruit body of PS1, the *nic*$^-$ and *nic*$^+$ gene segregated and the reoccuring of *nic*$^-$ gene in the progenies as the recombinant type became possible. Actually, all recombinant types of PS1 progenies showed to be nicotin requiring. Considering the linkage relationship of the two genes in either of the two nuclei in each mycelial cell of PS1, the two genes might exist either in same chromosome or different chromosomes. It was because the two genes might not be in allelic forms. Moreover, the linkage relationship of the two genes in the two PS1 nucli might either be the same or difference. Therefore, we could not expect any linkage relationship between the two nicotine genes in either PS1 nuclei.

For the drug resistance markers, about 12 to 14 % of the progenies were showed to be acriflavin sensitive and 2 to 8 % of them showed to be able to

oxidized guaiacol into brown product. These two populations of progenies showed to have the phenotypic character of Pf67 rather than that of Sc17. Although most wood rotting fungi were expected to be able to produce a lot of laccase and hence became able to oxidize guaiacol into brown products, the monokaryons of the *Schizophyllum commune* was reported to has a low ability in producing laccase (De Vries *et al.*, 1986). In fact, the Sc17 was tested to be unable to oxidize guaiacol in brown product. Therefore, the two phenotypes might be due to the presence of Pf67 genetic materials in the corresponding progenies. Hence, it was an indirect evidence for the existing of Pf67 genetic materials in fusion product PS1.

Although the unexplainable segregation pattern of auxotrophic and biochemical markers were found in the progenies of PS1, the segregation of incompatibility factors of the two PS1 nucleus was found to followed the Mendelian pattern (table 7.8). As the mating type of M4 type regenerants showed to be the same as that of Sc17, both A and B incompatibililty factors of M9 type nucleus were different from that of the former one (table 7.7.). On the other hand, the formation of fruit bodies and true clamp connections indicated that the two sets of incompatibilty factors in PS1 were different ($A \neq B \neq$) and in allelic form. Therefore, the Sc17 genetic material might exist in M9 type nucleus. However, some kinds of change might occur and caused mutation(s) in both A- and B-incompatibility factors. We still did not know the source of such mutation and one of the possibility was the synkaryotic nature of this nucleus which was resulting from the interaction of the genetic materials of the genomes of the two fusion parents.

For PS2, it showed to be able to produce both adenine and nicotine. For PS3, although the morphological character was very similar to that of Pf67, PS3 showed

to be able to produce adenine as well as acriflavin resistance. Therefore, both strains showed to have a mixed phenotype of the two fusion parents. If these reverse mutations were caused by the interaction of the genome of the two fusion parents, the fusion products PS2 and PS3 might be synkaryons.

In summary, the results of AP-PCR generated genomic fingerprinting and progeny analysis showed some information on the genetical difference between the three fusion products and the two fusion parents. However, based on the presence knowledge of genetics, we still could not explain all the phenomenon mentioned above. For solving the problems of the exact mechanisms that causing the existing of those phenomenons, we still have to wait until we have more knowledge on the interaction of genetic material of the in intergeneric protoplast fusion.

Part IV

Summing-up

Chapter 8

General Summary and Conclusion Remarks

8.1. General summary

Breeding of academic and commercial strains of basidiomycetes, especially for the edible mushroom, using the technique of hyphal-crossing between mycelia of mating types is only applicable in intraspecific hybridization. Protoplast fusion techniques provided some alternative approaches for obtaining interspecific and intergeneric hybrids. Hence hybridization through protoplast fusion has the principle advantage of hybridizing incompatible strains. Such kind of artificial "outbreeding" techniques thus created a novel route for bringing genomes into the same cytoplasm. Genetic variations in the genome of the fusion product relative to those of the fusion parents may result. Successful intergeneric protoplast fusion programmes have been reported mainly for some commercial fermentation strains such as *Aspergillus* and *Monascus* species (Kiyohara *et al.*, 1990.), *Aspergillus* and *Trichoderma* species (Kirimura *et al.*, 1989) and *Saccharomyces* and *Zygosaccharomyces* species (Pina *et al.*, 1986). Only a few intergeneric protoplast fusion programmes dealing with basidiomycetes have been reported (table 2.2.). In the presence study, we have attempted to carry out an intergeneric protoplast fusion experiment of two basidiomycetes, *Pleurotus florida* (Pf67) and *Schizophyllum commune* (Sc17) through PEG-mediated fusion method.

This thesis can be broadly divided into three parts. The first part involved the studies dealing with the investigation of the specific conditions for protoplasting of the two fusion parents. For optimization of protoplast isolation conditions such as the specific types of cell wall lytic enzyme(s) and cell wall digestion duration as well as the type and concentration of osmotic stabilizer for the fusion parents were studied. It was found that the protoplast release condition of the two fusion parents strains were mostly different. Although experimental conditions for protoplast release of the same species of the two fusion parents has been described by other biologist, the established protoplast release conditions were specific for the two fusion parents strains.

The second parts involved the study on the parameters for assessing the effect of different experimental steps of the protoplast fusion procedure on the two fusion parents. For assessing the protoplast fusion procedure, the percentage of nucleated protoplasts, protoplast regeneration frequencies and the stability of selective markers throughout the fusion process were studied. Although treatment of polyethylene glycol (PEG) on protoplasts only caused a statistically insignificant decrease in the regeneration frequency of the two strains, the regeneration frequencies of the untreated protoplast were low for both of the two fusion parent strains. Considering the effect of PEG on the stability of genetic markers, the auxotrophic as well as the drug resistance markers of the two strains found to be stable in the presence protoplast fusion system.

The third part involved the characterization of the fusion products obtained in this protoplast fusion system. Comparisons made between the fusion products and the fusion parents were based on their macroscopic and microscopic morphologies of vegetative mycelium and the corresponding fruit bodies, the growth response to

drugs as well as their corresponding genomic fingerprints. Progeny analysis was also carried out for the dikaryotic fusion product. The experimental results showed that the fusion frequency of the present intergeneric protoplast fusion system is rather low. Nevertheless, totally three fusion products, one dikaryon and two monokaryons, were obtained separately from three fusion experiments. Among the four AP-PCR genomic fingerprint profiles for each fusion product, two fusion products (PS1 and PS2) showed to have some fingerprint profile similar to that of single fusion parents (Sc17 and Pf67 respectively). However, all fingerprint profiles of PS2 showed to be completely different from that of both fusion parents. None of them showed to have a "mixed" fingerprint profile of the two fusion parents. These results indicated that part of the PS1 and PS3 genomes likely to be originated from single fusion parent. However, changes in DNA sequences reflected by the variation in their corresponding fingerprint profiles was alos observed. For PS2, the result only indicated that its genomic content was different from that of the two fusion parents. Hence, the present intergeneric fusion system may be able to cause some kinds of genetical changes in either fusion parents. However, the exact nature and mechanism responsible for such genetical changes was unknown. Besides the relative changes in genetical contents in the three fusion products were observed, results of morphological studies also showed that fusion products were partly similar to one of the fusion parents. Fruit body of the dikaryotic fusion product also showed to have a *MED* mutant phenotype. However, most mature basidia were tetrasporic. Variations occurred not only in the morphological aspect but also on the aspect of growth rate and the sensitivity to two drugs (acriflavin and guaiacol). Relative increases in growth rates were observed for all three fusion product corresponding to their morphologically similar fusion parents. On the other hand, significant increases in resistance to acriflavin were observed for all three fusion products. For the progeny analysis of the dikaryotic fusion product PS1, the reoccurrence of parental

(Sc17) nicotine requiring auxotrophic marker in the recombinant type progenies was rather interesting. The nature of "masking" mechanism for the nicotin requiring auxotrophic marker in both nuclei of PS1 was unknown. These analyses further indicated that the fusion products were atypical of the two parental strains.

8.2. Concluding remarks and further studies

Intergeneric protoplast fusion of *Pleurotus florida* and *Schizophyllum commune* was carried out as an attempt to generate novel hybrids. The morphological, physiological as well as genetical characters of three fusion products showed to be atypical to the two fusion parents. Evidence for the existing of genomic material of single fusion parent in two of the three fusion products was obtained in AP-PCR fingerprinting technique. No direct evidence was obtained for the coexisting of the both fusion parents' genomes in single fusion products. Therefore, the present intergeneric protoplast fusion system was able to cause some changes, both genetical and phenotypical, to the fusion parents and manifest these changes in the fusion products. However, it is too early to claim for the generation of intergeneric hybrids because of the complex incompatibility mechanisms that operate and control the interaction of the two genomes at the cytoplasmic and nuclear levels. In addition, at present, the genetic and taxonomical relationships, including the degree of chromosomal homology, between *P. florida* and *S. commune* remain poorly understood. Moreover, the expression of fruiting ability by the dikaryotic fusion product PS1 also open up a questionable area in the true classification at genus level for the two fusion parents.

For the present study, further research may be focusing on the following three aspects. Firstly, more rapid and accurate as well as efficient should be used for

screening the possible hybrids. Although AP-PCR fingerprinting is one of the rapid screening method for the present purpose, result of DNA profile comparison did not provide enough information for comparing the DNA sequence homology between the fusion products and the fusion parents. For the purpose to compare the DNA sequence homology, application of AP-PCR may be changed for obtaining a strain specific set of DNA markers for the two fusion parents. Therefore, these AP-PCR generated DNA markers can be used as probes for generating the specific hybridization pattern for the fusion products. Hence, by hybridization of the radioactive labeled AP-PCR generated parental DNA markers with the partial digested genomic DNA, possible hybrids can be screened out from numerous fusion products. However, it is of great importance to have a control experiment for investigating the hybridized patterns generated by the hybridization of the specific set of probes with the DNA digest of both of the two fusion parent strains.

Secondly, further study may be focused on the meiotic division event occurred in the basidia of the fruit body of PS1. Although tetrasporal basidia were shown in the electron micrographs in the present study, we got no information of the nuclear division event happening inside. The importance of studies on this aspect was based on the fact that the occurrence of complete meiotic division process in basidium was characterized for the true sexual reproduction of the dikaryon. Cytological study of nuclear division event can be carried out by DAPI nuclei stain as described in section 5.2.3..

Thirdly, it is desirable for obtaining more fusion product in order to have a more representative analysis of the present protoplast fusion system. Therefore, the conditions for protoplast fusion of the present fusion system may be optimized. As the specific protoplast releasing conditions for the two fusion parents have already

optimized, the study should therefore be focusing on the effect of aspects on different types and concentrations of polyethylene glycol, the optimum pH and the optimum concentration of calcium ion using in this fusion system.

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APPENDIX

SOLUTIONS

Chloroform	:	*[UNIVAR]
Chloroform : TE-saturated phenol	:	1:1, v:v
DAPI dye [Sigma] solution	:	0.5 μg ml^{-1} DAPI (4',6-diamidino-2-phenylindole) in McIlvaine's citrate / phosphate buffer pH 7.0
dNTPs	:	[PERKIN ELMER CETUS] 10 mM deoxyadenosine triphosphate 10 mM deoxythymidine triphosphate 10 mM deoxycytidine triphosphate 10 mM deoxyguanosine triphosphate
70 % Ethanol	:	70 % absolute ethanol [MERCK] 30 % autoclaved double distilled water
0.5 M Glycine/ sodium hydroxide 7.5 buffer pH 7.5	:	0.5 M glycine [SIGMA] dissolved in distilled waster adjust pH with 4 M sodium hydroxide [SIGMA] to pH
Glutaraldehyde fixation solution	:	6% glutaraldehyde [SIGMA] 0.1 M Sorenzen's phosphate buffer pH7.2 0.1 mM Magnesium sulphate 10 mM Sucrose
Isopropanol	:	[MERCK]
Loading buffer (DNA)	:	0.25 % bromophenol blue [BIORAD] 0.25 % xylene cyanol FF [BIORAD] 15 % Ficoll (Type 400; [Pharmacia]) in water
Lysis buffer	:	50 mM Tris-HCl (pH 7.2) 50 mM EDTA [SIGMA]

		3 % SDS [SIGMA] 1 % 2-mercaptoethanol [SIGMA]
Mycelium fixation solution	:	Ethanol : acetic acid = 3 : 1 (v : v)
McIlvaine's citrate / phosphate buffer pH 7.0	:	0.1 M citrate ($C_6H_8O_7$) : 0.2 M $Na_2HPO_4 \cdot 2H_2O$ = 1 : 5.66 (v : v)
3M NaOAc [UNIVAR]	:	3 M $NaOAc \cdot 3H_2O$ Adjust the pH to 5.2 with glacial acetic acid
10 X PCR Buffer II (Premixed)	:	[PERKIN ELMER CETUS] 500 mM KCl 100 mM Tris-HCl (pH 8.3)
PEG solution	:	35% w/v Polyethylene glycol [SIGMA] 4,000 0.05 M Glycine / sodium hydroxide buffer pH 7.5 0.05 M Calcium chloride [SIGMA]
RNAase solution	:	10 mg/ml pancreatic RNAase (RNAase A) [SIGMA] in 0.01 M sodium acetate [SIGMA] (pH 5.2) (Heat to 100 ^{o}C for 15 minutes and store at -20 ^{o}C)
Sorenzen's phosphate buffer pH 5.8	:	0.83 g Monopotassium dihydrogen phosphate 0.096 g Dihydrated disodium hydrogen phosphate In 100 ml buffer
Sorenzen's phosphate buffer pH 7.2	:	0.26 g Monopotassium dihydrogen phosphate 0.85 g Dihydrated disodium hydrogen phosphate In 100 ml buffer
10 X TBE	:	0.9 M Tris [MERCK] 0.9 M Borate [SIGMA] 0.02M EDTA
TE	:	10 mM Tris-HCl (pH 8.0) 1 mM EDTA (pH 8.0)
TE (for resuspending DNA pellet)	:	10mM Tris-HCl, 0.1 mM EDTA

1M Tris-HCl pH 7.2	:	1 M Tris Adjust the pH to 7.2 by concentrated HCl
1M Tris-HCl pH 8.0	:	1 M Tris Adjust the pH to 8.0 by concentrated HCl
1M Tris-HCl pH 8.3	:	1 M Tris Adjust the pH to 8.3 by concentrated HCl

*REMARK : [ ] - Brand name of the solution.